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# AFLATOXIN ELIMINATION WORKSHOP
Fresno, California, November 1-3, 1992

Aflatoxin is recognized as a serious food safety hazard by most countries of the world. Producing food free of aflatoxin today requires a truly national effort and, particularly, the cooperation of both government and industry. The Agricultural Research Service and the commodity groups representing peanuts, corn, cottonseed and tree nuts recognize the importance of a strong national research effort to eliminate aflatoxin as a food safety threat.

This Aflatoxin Elimination Workshop, held in Fresno, California, is the fifth such yearly meeting held to review the ARS supported aflatoxin research and provide a forum for interested scientists to come together to discuss common problems and their potential solutions among themselves and with members of the industry. Thus although many of these scientists are performing very fundamental studies they gain a very clear idea of where their research is leading and the impact it will have on society. Also, this workshop provides the opportunity for gains in cost effectiveness of research by the recognition of common approaches and by sharing relevant information across commodities. This workshop has come to be recognized as the premier national meeting for advances leading to methods to eliminate aflatoxin.

Most of the research is performed by the ARS, however an important addition to this core effort is provided through a competitive award program provided by Congressional appropriations. This program is a unique effort of the ARS and representatives of the peanut, corn, cotton and tree nut industries. By extending the opportunity for the best university scientists to join the highly focused multithrust program, the rate of progress toward the elimination of aflatoxin is enhanced.

On the following pages are the abstracts of work presented at the 1992 Workshop.

Jane Robens

Jane F. Robens
Agricultural Research Service
Workshop Program Coordinator

December 7, 1992

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ABSTRACTS OF PLATFORM PRESENTATIONS, POSTERS AND PANEL DISCUSSIONS

AFLATOXIN ELIMINATION WORKSHOP

Fresno, California, November 1-3, 1992



* Name of First Author only is included for all abstracts.

# Distribution of Aflatoxin in Pistachios

Thomas F. Schatzki[1]

We have derived a relation between the distribution of aflatoxin concentration among sample nuts in a lot, f(c), and the resulting distribution of aflatoxin concentration, C, among samples of n nuts, F(n,C). The relation is designed for rare infestation, common in pistachios and other tree and ground nuts. It points out that sample size is as important as concentration in characterizing a lot. In the limit of very rare infestation only a single nut infested nut will be present in the sample, resulting in F(n,C) = f(c)*n, while C = c/n. Heavier infestation, where several nuts are infested in a sample, lead to more complex relations between F and f. Using these functions one may thus compute the probability of having a lot accepted, based on a sample of one size, given only knowledge about samples of another size. One is aware that different regulatory agencies require different sample sizes.

A number of sample studies and surveys of aflatoxin concentration C, done at various sample size over the last 12 years, were collected and analyzed. After reduction to the underlying nut distribution f(c) one can arrive the following conclusions.

1. Aflatoxin contamination in market-ready pistachios is extremely rare, occurring in but 1 nut per 900 lb at the action level or 1 in 200 lb at the detection level. Such frequencies are subject to Poisson statistics and analytic results are not indicative of lot content. All results should be reported to include sample size, which is as important as actual aflatoxin level.

2. The various long range studies and laboratory records, compiled during the last 12 years, are consistent. There is little or no evidence of an effect of processing. Year-to-year levels, while fluctuating 2-fold or more, show no long term trend.

3. Sorting reduces aflatoxin levels around 4-fold at most concentration levels. The effectiveness of removing very highly contaminated nuts, which are responsible for the bulk of the total aflatoxin, is not yet clear.

4. Aflatoxin level of imported in-shell product is roughly 50% higher that of domestic product, but shell stock is 4 times higher. However, sample is limited and thus confidence of these results is low.

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# Evaluation of Characteristics of Pistachio Nuts for Use in Aflatoxin Elimination

**Mark A. Doster and Themis J. Michailides**, Department of Plant Pathology, Univ. of California, Davis/Kearney Ag. Center, Parlier, CA 93648

In 1986, it was shown that early split (ES) pistachios (abnormal nuts that have the hull and shell split exposing the kernel to invasion by molds and insects) contained most of the aflatoxins. However, no studies have been done to evaluate specific characteristics of pistachio nuts that could be used to separate ES from normal nuts during processing. In 1991, we observed that many ES from commercial pistachio orchards had shriveled hulls. Last winter when we analyzed samples of pistachio nuts for aflatoxins, we found that 31% of the samples of shriveled ES had aflatoxins with an average of 31 ppb, while only 6% of the smooth ES samples had aflatoxins with an average of 0.4 ppb. As expected, none of the samples of normal nuts that had nonsplit hulls were contaminated with aflatoxins. From the 1992 harvest, we found that 2.5% of the shriveled ES were infected with _Aspergillus_ _flavus/parasiticus_, while only 0.5% of the smooth ES were. The distinctive appearance of the shriveled ES should allow easy separation from the normal nuts with smooth hulls.

In addition, in 1992 we discovered that ES did not occur all at once, but instead occurred starting in mid July and continuing throughout August. The old ES (formed in July and early August) were found to have substantially more _Aspergillus_ infections in the kernel than the younger ES (formed in late August) and to be very distinct from normal nuts in staining, size, weight, and moisture content. In one commercial orchard, the older ES compared with normal nuts had smaller shells (mean length 17 mm for ES and 23 mm for normal), lighter fruit (fresh weight 1.3 g versus 4.0 g), lighter kernels (dry weight 0.5 g versus 1.4 g), and lower kernel moisture content (20% versus 51%). Older ES were more likely to have shriveled hulls.

The staining of the shell may be useful for separating out nuts contaminated with aflatoxin. We found that ES typically have a distinctive dark staining along the shell suture. This suture staining that is characteristic of ES has been observed in commercial processed nuts offered in retail outlets. In addition, ES can have a general dark staining on part of the shell surface. The older ES were found to be more likely to have the typical suture staining and more general staining than the younger ES or normal nuts.

Several characteristics (shriveling of hull, size, weight, moisture content, and shell staining) were different for those nuts likely to have aflatoxins when compared with normal healthy nuts. The use of these characteristics would allow removal of contaminated nuts and would result in a substantial reduction of aflatoxins in processed pistachio nuts.

# Separating "Early-Split" from "Normal" Pistachio Nuts for Removal of Nuts Contaminated on the Tree with Aflatoxin.

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**Summary:**

A research project was initiated to develop a method of separating "early-split" from "normal" pistachio nuts to remove nuts that are infected with *Aspergillus flavus* and possibly contaminated with aflatoxin. Thompson and Mehdy[1] and Sommer et al.[2] observed that California pistachio nuts with hulls that split prior to harvest are much more likely to be infected with *A. flavus* than normal nuts. The objective of this research is to remove early-split pistachio nuts, which usually represent one to five percent of the pistachio nuts in California orchards at harvest, from the rest of the California pistachio crop resulting in a pistachio crop that is essentially free of aflatoxin.

The preliminary findings of this study are as follows. Food grade dye does not appear to be useful as a method of marking early-split pistachio nuts because the dye easily penetrates the hull in non-split nuts. Since the hull is often loose around non-split nuts, the dye can easily stain the whole nut once it gets through the hull. A large percentage of early-split pistachios were found to have a very noticeable brown stain around the split in the shell and their shells tend to have a darker yellowish shade to them. From 100 nut samples, it was found that 96% of early-split pistachio nuts, 28% of growth-split pistachio nuts, and 13% of non-split pistachio nuts had either a stain around the shell split and/or had the yellow discoloration. The hulls of non-split and tattered pistachio nuts were found to be much easier to remove than the hulls of early-split pistachio nuts. A mechanical device was developed to differentially hull pistachio nuts. Preliminary results indicated that all of the hulls on non-split and tattered pistachio nuts were removed while the hulls of early-split pistachio nuts were left intact. A second mechanical device using rolling needles was developed that removed 90% to 95% of early-split and tattered pistachio nuts from the non-split nuts with only 5% of the non-split nuts being removed during the process. The following physical properties of early-split, tattered and non-split pistachio nuts were determined during the 1992 harvest:

Length of Nut[3]
Height of Nut[3]
Width of Nut[3]
Mass of Nut[3]
Volume of Nut[3]
Density of Nut[3]
Moisture Content of Nut[3]
Terminal Velocity of Nut[3]

Hull Friction Factor
Hull Split Length[5]
Hull Split Width[5]
Hull Thickness
Hull Moisture Content

Mass of Nut[4]
Moisture content of Nut[4]
Shell Length
Shell Height
Shell Width
Width of Split in Shell

**Future Plans:**

Physical properties distinguishing early-split pistachio nuts from "Normal" pistachio nuts will be investigated to determine other possible methods of removing early-split nuts. In addition, computer vision techniques will be investigated to determine the feasibility of identifying early-split pistachio nuts using a machine vision sorting system.

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[1] Thomson, S.V., and M.C. Mehdy. 1978. Occurrence of aspergillus flavus in pistachio nut prior to harvest. Phytopathology. 68:1112-1114.

[2] Sommer, N.F., J.R. Buchanan, and R.J. Fortlage. 1986. Relation of early splitting and tattering of pistachio nuts to aflatoxin in the orchard. Phytopathology. 76:692-694.

[3] Unhulled

[4] Shell and kernel only

[5] Early-split nuts only

## U.S. Cotton Production and Aspergillus flavus.

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In cooperation with the National Cottonseed Products Association and member oil mills, we extensively sampled the 1991 cottonseed crop and analyzed the magnitude of the Aspergillus flavus population associated with it. The number of propagules of A. flavus per gram of cottonseed was found to differ among mills and among cotton growing regions. Some mills also provided data on the incidence of aflatoxin in seed received during 1991. Regions experiencing serious contamination problems (i.e. south Texas) and those with little or no contamination (ie. California) were identified by propagule counts. The results provide a picture of the relative exposure to potential contamination experienced by different components of the 1991 crop. Surveys such as this may effectively supplement aflatoxin data to provide a more comprehensive understanding of the relationship of A. flavus populations to cotton production. Such surveys may become useful tools for assessing the impact of changing agronomic practice and environmental conditions on A. flavus populations and for mapping the distribution of crop exposure to A. flavus between years. The resulting insights may permit modification of cultural practices to reduce crop exposure to A. flavus.

Within areas experiencing significant episodes of aflatoxin contamination, there is not always a simple correlation between the quantity of aflatoxin within the crop and either the percent of that crop infected by Aspergillus flavus or the quantity of A. flavus propagules associated with the crop. This lack of correlation can partially be attributed to both production of propagules in saprophytic niches and the fact that not all infections result in similar levels of contamination. However, when sampling extends over broad areas, propagule counts may be a useful tool for assessing relative crop exposure to A. flavus because, during periods favoring contamination, A. flavus populations increase rapidly as the cotton crop is produced. Propagule counts allow insight into the exposure of portions of the crop not routinely assayed for aflatoxins and provide greater sensitivity than aflatoxin tests. Propagule counts also are less variable than toxin measurements due to partial homogenization caused by propagule dispersal during various agronomic practices and post harvest handling.

A comprehensive understanding of A. flavus populations associated with cotton production will facilitate our efforts to modify A. flavus populations with atoxigenic strains. Estimates of both the magnitude of the A. flavus population associated with cotton production and the distribution of various toxigenic and atoxigenic A. flavus group strains and species are being developed. The relationship of environment and geographical locale to A. flavus population structure is being assessed, as is the impact of this structure on the contamination process.

# Aflatoxin Contamination of Peanut: Interaction of Environmental Variables, Soil Moisture, and Soil Insects

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Collaborative research between scientists at the USDA Insect Biology Laboratory, University of Georgia Mycotoxin Laboratory, and the University of Ouagadougou, Burkina Faso, West Africa, has been conducted to study the relationship between insect damage to peanut pods and aflatoxin contamination. Several cultural and environmental factors interact to enhance insect damage to peanut pods and aflatoxin contamination in peanut seeds. These factors include peanut cultivar, planting date, rainfall, distribution of rain, drought during the latter portion of the growing season, pod damage by soil insects, harvest date, drying conditions after digging, and storage conditions. In the U.S., research has focused on the lesser cornstalk borer (LCB) and in Burkina Faso on termites. Damage to peanuts by both of these insects occurs primarily during the latter portion of the growing season during drought, i.e. conditions that also favor Aspergillus flavus infection and aflatoxin formation in peanut seed.

In laboratory studies, LCB larvae were an excellent vector of an A. parasiticus color mutant to all developmental stages of peanut pods. Fungal invasion and aflatoxin concentration in seed were greater in immature pods (stages 2-3) than in more mature pods (stages 4-6). Contamination of seed was directly related to the extent of pod injury by the insect larvae.

In field studies, over 50% of the LCB larvae collected from peanuts were naturally contaminated with species of the A. flavus group. Increased pod injury by the LCB increased the percentage of seed contaminated with A. flavus. Seed from pods with only external scarification due to insect feeding had a significantly higher percentage of A. flavus infection than seeds from uninjured pods, but no difference was noted in aflatoxin.

Research under rain-out shelters where peanuts were grown under optimum conditions for the first 90 days and then exposed to drought and infested with neonate LCB larvae showed a significant increase in A. flavus contamination and aflatoxin in seed from externally scarified pods compared with seed from undamaged pods. Likewise, seed from penetrated pods had significantly more A. flavus and aflatoxin than seed from externally damaged pods. The increase in A. flavus and aflatoxin is related to a more rapid water loss in pods with external scarification which allows seed in the damaged pods to reach a moisture content conducive to A. flavus growth and aflatoxin formation more rapidly than in seed from undamaged pods.

Research in Burkina Faso has shown that termite damage to peanut pods is one of the primary variables associated with aflatoxin contamination of the seed. Termite damage to peanut pods is very similar to that caused by LCB larvae in that damage is characterized by both external scarification and pod penetration. Termite damage to pods increases rapidly with drought during the latter portion of the growing season. With a definite rainy and dry season, termite damage can be greatly accentuated by a 14-day delay in harvest beyond that which is optimum for a given locale. The increase in termite damage is directly associated with a decrease in soil moisture during the latter portion of the growing season and coincides with an increase in contamination of seed with aflatoxin. Evaluation of peanut lines in Burkina Faso that rated resistant to termite damage in India has shown that several lines, notably NCAc 343, have resistance to both plant and pod damage due to termites. Research is presently underway to determine if peanut cultivars with resistance to termite damage are also resistant to the LCB and if the resistance to pod damage will decrease aflatoxin contamination.

Table 1. Aflatoxin concentrations (ppb) in corn from randomly selected cornfields in and around Tift County, Georgia, in 1987-1992.

| | Irrigated | | Non-irrigated | | Mean | |
|---|---|---|---|---|---|---|
| | No. Fields | Total ppb | No. Fields | Total ppb | No. Fields | Total ppb |
| 1987 | 20 | 85.0 | 20 | 78.0 | - | 81.5 |
| 1988 | 17 | 42.0 | 26 | 200.0 | - | 137.2 |
| 198[illegible] | 2[illegible] | 5.0 | 22 | 46.0 | - | 25.5 |
| 1990 | 15 | 100.0 | 30 | 277.0 | - | 217.8 |
| 1991[1] | - | - | - | - | 45 | 38.9 |
| 1992[1*] | - | - | - | - | 40 | 35.6 |

1 Due to adequate rainfall during this year, no distinction was made between irrigated and non-irrigated fields.

* Grain samples were collected at plant maturity during the third week of August.

# Aflatoxin Early Warning Systems

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New devices that will be available in the near future are "aflatoxin early warning systems". They are placed in a peanut farmer's field and can predict how much drought stress the peanut crop can endure and still be free of aflatoxin. The systems provide the peanut farmer with a management option of harvesting his peanut crop early to ensure that it remains free of aflatoxin while possibly compromising, to some extent, other quality factors such as maturity. The peanut farmer may decide not to harvest early but to wait for rainfall. However, if adequate rainfall does not occur, he will harvest a poorer quality peanut crop that will be contaminated with aflatoxin.

We are conducting research on two separate early warning systems. One is an expert system that was developed in conjunction with Neogen Corporation. An instrument (Enviro-Caster) is currently in the advanced stages of development with commercialization possible next year.

The second system is being developed in conjunction with Dr. Bailey Mitchell, an Agricultural Engineer with ARS Poultry Laboratory in Athens, Georgia. It is based on a computer prediction model that predicts levels of aflatoxin based on rainfall and soil temperature. A patent is being drafted and, if all goes well, it should be available commercially in the next 2-3 years.

Reference

Thai, C. N., P. D. Blankenship, R. J. Cole, T. H. Sanders and J. W. Dorner. Relationship between aflatoxin production and soil temperature for peanuts under drought stress. Trans. Am. Soc. Agric. Eng. 33:324-329, 1990.

## *Aspergillus* incidence, Aflatoxins and Interactive Effects of Lesser Cornstalk Borers in Peanuts

by K.L. Bowen and T.P. Mack

The ability of larvae of the lesser cornstalk borer, *Elasmopalpus lignosellus* (Zeller), to augment contamination of peanut pods with aflatoxigenic fungi, *Aspergillus flavus* Link and *A. parasiticus* Speare (*A. flavus*-type fungi), was investigated in laboratory and field studies. Aflatoxigenic fungi were found in or on frass from 28.6% of field-collected larvae and in 8.9% of sterilized and macerated larvae. More aflatoxigenic fungi tended to be found in pods from untreated plots than in plots treated with chlorpyrifos in field trials. Contamination of pods or seeds with *A. flavus*-type fungi was positively correlated in all four trials with scarification of pods, and this relationship is approximated by the model: $Afl = 2.37 + 0.0057\,(\%\ Scar^2)$ (see attached Fig.). Since appropriate insecticide treatments can decrease populations of lesser cornstalk borers, which would decrease pod scarification, these same treatments may decrease contamination with aflatoxigenic fungi. Treatment thresholds for the lesser cornstalk borer need to be reconsidered based upon this information. Field trials with insecticides, conducted in 1992, had minimal population of lesser cornstalk borers due to adequate rainfall levels, and incidence of *A. flavus*-type fungi in developing pods was lower than observed in previous two years.

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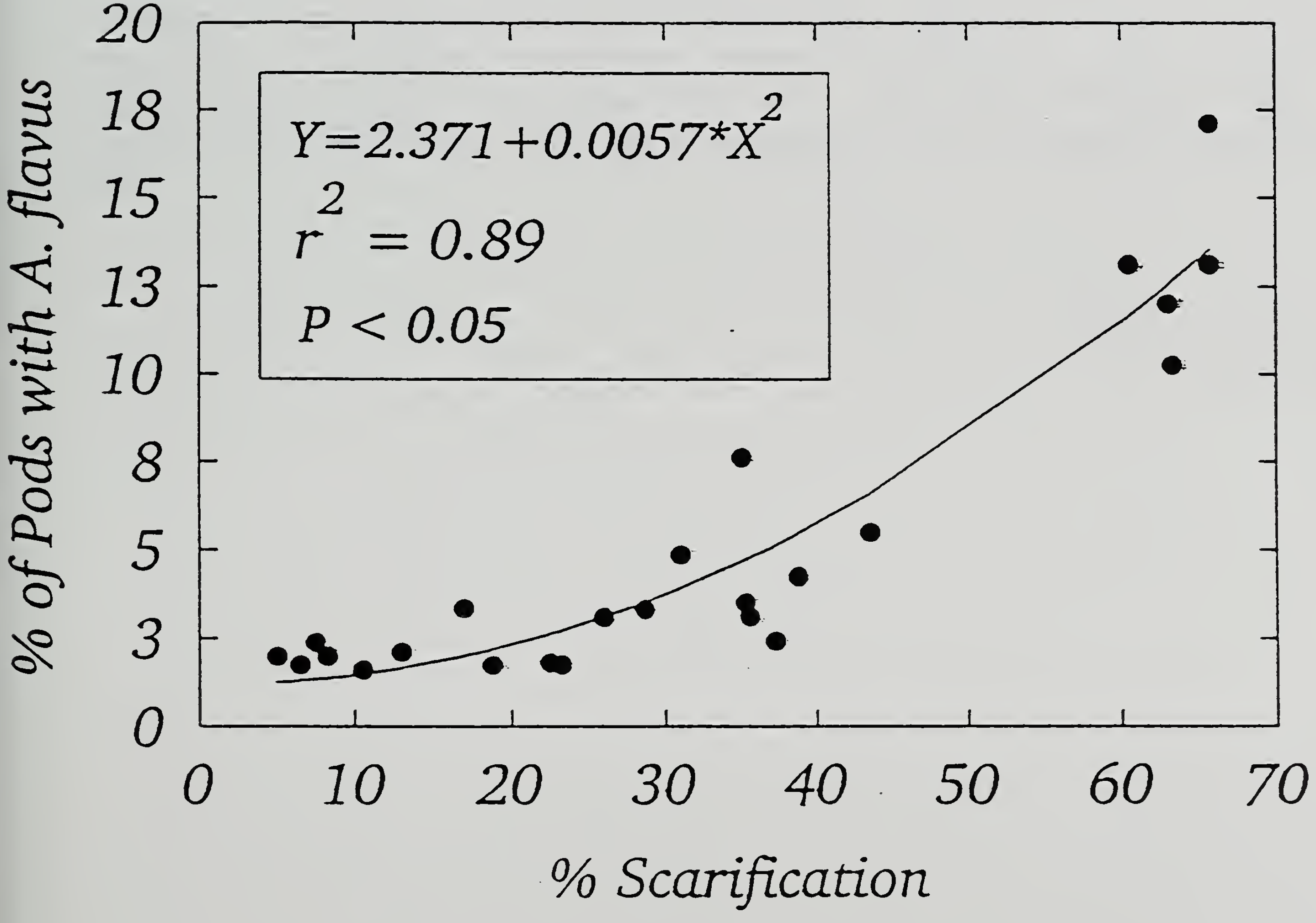
USDA, Food Safety & Health
Preharvest Control of Aflatoxin
Proposal Funded 4/91


20
18
15
13
10
8
5
3
0
% of Pods with A. flavus
Y=2.371+0.0057*X^2
r^2 = 0.89
P < 0.05
0
10
20
30
40
50
60
70
% Scarification

**Aflatoxin Concentration in Peanuts as Affected by Calcium and Boron**

by J.F.Adams, K.L. Bowen and C.J. Mickler

Calcium and boron deficiencies can affect the biological integrity of peanuts. *Aspergillus flavus* invasion of seed and subsequent aflatoxin contamination is more likely to occur when the seed integrity is compromised. Thus, peanuts produced under calcium and/or boron deficiencies may be more likely to be contaminated with aflatoxins. Preliminary data was collected from three on-farm experiments conducted in 1991. Experiments consisted of gypsum and no gypsum treatments with 4 replications using 'Florunner', 'GK 7' and 'Sunrunner' cultivars. Yields did not increase with applied gypsum, but calcium concentration and germination percentages of harvested nuts did increase. The incidence of *A. flavus* in these nuts decreased with increasing soil calcium concentrations and increased with increasing soil pH. Subsequently, four on-farm experiments were initiated in spring of 1992. There were two calcium and two boron experiments. The calcium experiments consisted of 'Florunner', 'GK 7', and 'Sunrunner' cultivars with no gypsum and gypsum treatments. The boron experiments were conducted with 'Florunner' only. These experiments were not harvested until October of 1992.

USDA, Food Safety & Health
Preharvest Control of Aflatoxin
Proposal Funded 7/92

# Efficacy of Volclay NF-BC, Microfine Bentonite, to Diminish Aflatoxicosis in Rats

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Addition of sequestering agents to feeds and foods has been proposed as a protective strategy against mycotoxins found therein. To investigate the efficacy of Volclay, a bentonite clay, to protect against aflatoxicosis, rats were fed peanut butter (50% w/w)-based diets containing approximately 1500 ppb aflatoxin (AF), 1500 ppb aflatoxin with 0.1% Volclay supplementation (AF-LD) or 1500 ppb aflatoxin with 1% Volclay supplementation (AF-GD) for eight weeks. The control group was fed a peanut butter-based diet without aflatoxin or Volclay supplementation and a fifth group was fed the control diet with 1.0% Volclay supplementation (VC). No differences in appearance, behavior or selected hematological and serum chemical variables were found among groups. Decreased weight gain, decreased food consumption and liver lesions consistent with hepatic aflatoxicosis were found in AF-fed rats. Weight gain and food consumption of the AF-HD group were comparable to the control and VC groups and were significantly increased compared to AF-fed rats, even though weekly aflatoxin ingestion of AF-HD rats equaled or exceeded that of the AF group. Body weight and food consumption of the AF-LD group were slightly increased compared to AF group and decreased compared to the control, VC and AF-HD groups, but the differences were not statistically significant. Liver lesions were found in all AF and all AF-LD rats. Lesions were also found in eight of 10 AF-HD-fed rats, but were subtle and significantly less extensive than those found in AF and AF-LD rats. The data suggest that Volclay is nontoxic and may be an efficacious sequestering agent for residual aflatoxin found in peanut butter.

## Reference

Voss, K. A., J. W. Dorner and R. J. Cole. Amelioration of aflatoxicosis in rats by the sequestering agent Volclay NF-BC. Soc. of Toxicol. 1993 (Abstract).

10-21-92

An Integrated Approach for Controlling Sap Beetles and Mycotoxins in Corn

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Sap beetles (Coleoptera: Nitidulidae) can vector mycotoxigenic fungi, including Aspergillus flavus, to corn and other commodities. An integrated approach for controlling these insects should reduce levels of mycotoxins in corn. Such an approach, which depends on previously described methods, newly discovered strategies, and projected developments is as follows:

Cultural control, especially sanitation, can reduce beetle breeding areas and also reservoirs of mycotoxigenic fungi. This strategy has been used successfully for reducing sap beetle populations in different commodities.

Many natural enemies of sap beetles have been identified, including a new species of nematode discovered in Illinois. The effectiveness of these controls should be preserved by appropriate selection and timing of other control measures for sap beetles and other corn insects such as corn earworms.

Effective traps and attractants for sap beetles have been developed and can be used for monitoring and trapping out. Early spring trapping of emerging sap beetles should be delayed until infected insects (which are most common in April in central Illinois) have dispersed. "Perimeter defense" trapping has reduced levels of sap beetles and mycotoxigenic fungi in corn. Autoinoculating devices are being tested to disperse sap beetle pathogens, as well as biocompetitive microorganisms directly to wounded corn.

A single application of encapsulated malathion granules applied at 0.01 lb a.i. per acre in small plot trials significantly reduced sap beetles and caterpillar population levels in milk stage corn and the incidence of Fusarium spp. fungi in 1992 to levels comparable to five spray treatments with malathion at 1 lb a.i. per acre. This technique also prevents exposing natural enemies to malathion.

Corn ears should be physically protected from sap beetles through adequate husk coverage that still allows rapid drydown. Axels should not be "cupped" around the stalk to avoid pollen accumulation, which can become moldy and also become a food source for sap beetles. Silks should resist feeding by corn earworms, that can subsequently make holes allowing entry of sap beetles. Because husk coverage is decreased by drought, kernel resistance can be a second line of defense. Inbreds and hybrids cross-resistant to mycotoxigenic fungi, sap beetles, and other corn insects have been identified, but further investigation is needed. Rapid browning may be involved in kernel resistance. Parts of transgenic tobacco and tomato that brown rapidly were shown to be resistant to corn earworms and sap beetles in greenhouse tests in 1992. This mechanism could be incorporated into corn through genetic engineering or conventional breeding and promote cross-resistance.

Although more work is needed, especially in developing cross-resistant varieties, a framework for integrated control of sap beetles and mycotoxigenic fungi in corn exists. Further work will involve verifying and combining control measures into a comprehensive program for controlling sap beetles, other corn insects, and mycotoxigenic fungi.

"Relevant Publications - Past Two Years"

Bartelt, R. J., Dowd, P. F., Plattner, R. D. and Weisleder, D. Aggregation pheromone of dried-fruit beetle, *Carpophilus hemipterus*. Wind-tunnel bioassay and identification of two novel tetraene hydrocarbons. J. Chem. Ecol. 16:1015-1039. 1990.

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Kernel and Placental Infection in Maize Ears Field-inoculated with *Aspergillus flavus/parasiticus* Isolates in Mississippi

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The level of infection of *Aspergillus flavus* in whole kernels, kernels cut transversely into three segments, and associated placental (cob) tissue was determined in an attempt to establish the most probable infection route of this fungus in corn. Knowledge of the infection process will be helpful in developing new, more efficient inoculation techniques for screening maize genotypes for resistance to kernel infection by the fungus. When maize ears were field inoculated with six visually identifiable *A. flavus* type isolates, kernel infection, both for whole kernel and kernel parts, was generally three to five times greater than associated placental tissue infection. Although some fungal isolates were more aggressive than others, the infection pattern for all six fungal isolates with relation to infection of whole kernels/placentas or kernel parts/placental infection was similar. Chi square tests suggested that observed values for infection in both kernel and associated placental tissue were greater than would be expected by chance alone. Because the incidence of kernel infection is much higher than infection of placental tissue, most kernel infection by *A. flavus* probably takes place through the pericarp. Some fungal infection, however, can move from the kernel to the placenta or vice versa. Direct placental tissue infection may also occur, but probably has little, if any, influence on the incidence of kernel infection.

Effect of *Fusarium moniliforme* and *Aspergillus flavus* on Kernel Infection and Aflatoxin Contamination in Maize Ears

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*Fusarium moniliforme* is frequently recovered from symptomless maize kernels from ears inoculated in the field with *Aspergillus flavus* in Mississippi. When maize ears were inoculated simultaneously with *F. moniliforme* and *A. flavus* or with *A. flavus* alone in 1990, significantly fewer kernels were infected with *A. flavus* in ears inoculated with both fungi than kernels from ears inoculated with *A. flavus* alone. Grain from ears inoculated with both fungi had significantly less aflatoxin than grain from ears inoculated with *A. flavus* alone in two tests in 1990. Inoculation of ears with *A. flavus* alone in 1989 resulted in significantly more natural infection of kernels by *F. moniliforme*. In contrast, percentages of natural infection of kernels by *A. flavus* in ears inoculated with *F. moniliforme* alone and in uninoculated ears were both low and did not differ significantly. Apparently, *F. moniliforme* can inhibit kernel infection by *A. flavus* in inoculated maize ears and lead to reduced aflatoxin contamination in these kernels.

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# The Relationship of Interacting Biological and Environmental Stresses on the Susceptibility of Corn to Aflatoxin Contamination

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The Lower Rio Grande Valley of Texas has experienced severe outbreaks of aflatoxin contamination in corn crops during 3 out of the last 5 years. These outbreaks appear to be the result of marginally adapted hybrids exposed to disease and insect pressure during periods of environmental stress. In the fall of 1991, we began developing a detailed environmental data base in an attempt to improve our understanding of the relationship between water stress, heat stress, insect pressure, and cultivar susceptibility to aflatoxin contamination. The environmental data also provides year to year continuity needed to explain the role of environmental stresses in periodic outbreaks of aflatoxin contamination. Micrometeorological measurements of air temperature, solar irradiance, net radiation, soil heat flux, relative humidity, and wind speed were taken continuously during crop maturity with an automated data logger. Periodic measurements were made to determine soil moisture, rainfall, and canopy temperature. Infrared photography and thermal infrared imaging were used in the determinations of crop development and stress. Corn earworm (*Helicoverpa zea* [Boddie]) infestation, soil and airborne populations of *A. flavus* and relative toxigenicity of the soil populations were determined in irrigated and nonirrigated plots. Approximately 14 hybrids and crosses were screened for susceptibility to aflatoxin contamination by inoculation with spores from toxigenic strains of *A. flavus* isolated from local soils.

The fall crop had only low levels of aflatoxin in the control (<5 ppb) and inoculated (100 to 300 ppb) treatments. The spring crop was higher with the control at 7 to 342 ppb and the inoculated between 500 and 2275 ppb. Insect infestation ranged from 25 to 100% with no correlation to aflatoxin levels. Irrigation had no effect on the aflatoxin accumulation in either crop. Airborne spore loads were higher in the spring averaging approximately 97 colonies per $m^3$ of air. Soil populations also were higher in the spring yielding nearly 1000 colony forming units per g of soil as compared to 417 in the fall. The percentage of toxigenic isolates found in soil sampled during the spring was highest in nonirrigated plots (52% and 28%, nonirrigated and irrigated, respectively). Canopy temperatures and leaf senescence in the spring crop were reduced by irrigation during the last 4 weeks prior to harvest. Consequently, the irrigation treatments succeeded in reducing plant stress. However, the accumulation of aflatoxin was the same in irrigated and nonirrigated plots. Based on this limited study, increasing temperatures and evaporative demand late in kernel development were the only measurable factors associated with increasing aflatoxin contamination. We have identified a susceptible (NC+6414) and less susceptible (Asgrow 404) cultivar, however, other culitvars were considered to be less susceptible because of toxic silks (maysin) or performance in other locations. These cultivars all appeared to be susceptible when grown under climatic conditions typical of the Weslaco location. The least susceptible cultivars identified in both the fall and spring tests had tightly wrapped husks that may have protected the developing kernels from *A. flavus* and aflatoxin accumulation. We feel that increasing temperatures during kernel desiccation sets up conditions favorable for aflatoxin synthesis and accumulation. Supplemental irrigation is ineffective in overcoming these conditions favoring aflatoxin synthesis. Corn earworm damage offers a localized infection site but does not appear to have a direct function in triggering the synthesis of aflatoxin. Early spring planting, possibly a fall crop, early harvest coupled with grain drying, and tighter husked cultivars represent immediate options to explore for managing to reduce the risk of aflatoxin contamination. The consistent use of a detailed environmental data base is critical for separating "the possibilities" from facts in order to bring continuity into experiments conducted at different times and locations. Understanding the microenvironment surrounding developing kernels and the physiological conditions responsible for kernel susceptibility to *A. flavus* during heat stress are critical to identifying current hybrids and producing new cultivars with reduced susceptibility to aflatoxin contamination.

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# Monitoring Environmental Conditions and Crop Growth Associated with Aflatoxin Contamination in Corn

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Heat and drought stress often occur in conjunction with aflatoxin contamination in corn. However, the relationship of aflatoxin contamination to specific environmental conditions remains unclear. Therefore, plots were established near Weslaco, Texas, with instrumentation to gather detailed micrometerological data on moisture and temperature conditions associated with aflatoxin contamination in corn. Data was accumulated from irrigated and dryland plots. Five-minute averages of air temperature, relative humidity, wind speed, solar irradiance, net radiation, and soil heat flux were recorded using a data logger over the growing season. Daily rainfall was measured using a gauge adjacent to the study plots. Available soil moisture to a depth of 1 m was measured weekly from soil cores in each of the treatment plots. Leaf area index (LAI) and total live leaves per plant were measured as the crop developed and matured. Grain yield was determined from subsamples collected at harvest. Aerial infrared photographs and thermal infrared imaging radiometry were used to confirm the presence of plant stress in irrigated and dryland plots. Our results indicate that irrigation successfully reduced late season stress and delayed crop senescence. Despite the reduced stress, there were no differences in grain yield or aflatoxin contamination between the irrigated and dryland treatments. The least susceptible variety to aflatoxin contamination after inoculation and natural infection exhibited a tight husk phenotype. We are proposing that high evaporative demand during the last 25 to 30 days prior to harvest increases kernel susceptibility (physical and physiological) and favors the synthesis of aflatoxin by *A. flavus*. At this stage of development, supplemental irrigation is unable to moderate the effects of high evaporative demand and/or heat on the kernel. Our preliminary results with tighter husk covers imply that the environmental stress (high evaporative demand, heat) directly influences the microenvironment surrounding the kernels and their ability to suppress the accumulation of aflatoxin.

# Aflatoxin Resistance in Selected Corn Varieties as Affected by Corn Earworm Infestation

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Experiments were conducted to: 1) determine the importance of corn earworm in establishing infection sites for *A. flavus* and aflatoxin accumulation and 2) examine the chemical basis for varietal resistance to earworm infestation and susceptibility to aflatoxin contamination. Seven corn hybrids were planted at Weslaco, Texas during March of 1992. Silks from four hybrids (NC+6414, Wilson 1890, HS 64, Zapalote Chico x Mp 313E) were considered toxic to corn earworm, while two additional hybrids were included as aflatoxin resistant (Tx 801 x Tx 805) and aflatoxin susceptible (Tx 804 x Tx 814). Silks were inoculated with corn earworm eggs and/or *A. flavus* spores. All inoculated varieties were equally infested by corn earworm and were not different from the controls. NC+6414 and HS 64 were the most susceptible varieties to aflatoxin accumulation resulting from inoculation. Either damage by the corn earworm or presence of *A. flavus* or a combination of both, resulted in an increase of aflatoxin over the background level in the control group. With the exception of Tx 804 x Tx 814, feeding studies with silks from varieties considered to be toxic resulted in decreased corn earworm larval growth. However, similar antibiosis was not observed in the field. Using the procedure published by Gueldner et al.( J. Agric. Food Chem.,1992, 40:1211-1213), we were not able to unequivocally identify the presence of maysin in silk samples from any variety. High performance liquid chromatographic procedures are being implemented for the ongoing determination of maysin. In summary, the *A. flavus*/corn earworm silk inoculation treatment was effective in elevating aflatoxin contamination, though we were unable to separate the relative contributions of *A. flavus* and corn earworm. Four out of the seven varieties tested displayed reduced aflatoxin susceptibility to artificial (inoculated) and natural (control) infection. Varietal differences in silk antibiosis were identified with diet incorporation techniques and *in vitro* larval growth studies. However, the same varietal differences were not expressed as resistance to corn earworm in the field. Severe environmental (heat) and biological (intense insect pressure) stresses typical of the Weslaco location in addition to the inoculation procedure may have altered varietal differences in resistance to corn earworm and susceptibility to aflatoxin contamination previously identified at other locations.

# AFLATOXIN IN CORN IN NORTHEASTERN MEXICO: EFFECT OF ENVIRONMENT AND CULTURAL PRACTICES

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Corn has been cultivated in northern Tamaulipas, Mexico since the early 60's. Yields have fluctuated accordingly to climatic conditions and pest problems. Occurrence of *Aspergillus* in this area was sporadic until 1989, when contamination (>20 ppb) of grain was estimated at 98 %. In 1990, contamination was 92 %. In 1991 when a regional plan to minimize aflatoxin was adopted, contamination was 23 %. In 1992, contamination was <1 %.

The environmental conditions most associated with aflatoxin are heat and drought, particularly at flowering and kernel development. In 1989, when contamination was high, max and min temperatures during the critical stage (Abr 20- Jun 10) averaged 24 and 34 $^{o}$C, with only 32 mm of rainfall. These conditions were very similar in 1990 and 1991. In contrast, in 1992 when contamination was very low, min and max temperatures averaged 21 and 31 $^{o}$C, with 192 mm of rainfall, optimal conditions for corn growth, and inadequate for aflatoxin synthesis.

Observations in northern Tamaulipas during 1989 and 1990 and experiences from other corn regions offered the basis to adjust corn management to minimize aflatoxin. Modifications included planting earlier, reduce plant population (max 55,000 pl/ha), avoid drought stress by irrigating when necessary, and controlling ear insects (*H. zea* and *S. frugiperda*). Implementation of these changes produced a beneficial impact during the 1991 growing season with only 23 % of the production contaminated, despite climatic conditions (heat and drought) were very similar to 1989 and 1989, when contamination was >90 %.

A series of experiments was conducted during 1991 and 1992 to determine the impact of agronomic factors, including planting date, variety, irrigation, plant density, fertilization, ear insect control, and harvest date. Although the results are preliminary, the most important findings are: (a) planting dates later than February 15 have a higher risk of aflatoxin; (b) ear insects increase significantly aflatoxin contamination; (c) no differences among local commercial varieties have been detected; (d) the "INIFAP" management system (var H-422, early planting, 55,000 pl/ha, adequate irrigation, control of ear insects) has showed the highest yields and lowest aflatoxin levels; (e) a small proportion of the ears (2 %) infected with *Aspergillus* (> 300 ppb) contaminate the healthy ears (98 %) (< 8 ppb); similarly, aflatoxin in individual ears concentrates in those parts damaged by insects (417 ppb), in contrast to the undamaged parts of the same ears (7 ppb).

AFLATOXIN ELIMINATION WORKSHOP
November 1-4, 1992
Fresno, California

Panel Discussion: To What Degree Can Optimum Crop Production and Handling Practices Eliminate Aflatoxin?

Panel Members: T. Schatzki, T. Michailides, P. Cotty, P. Dowd, J. Dunlap, N. Zummo, and R. Lynch, Chair

Aflatoxin contamination of dried fruit, almonds, pistachios, walnuts, cottonseed, corn and peanut continues to be a great concern to both individual commodity groups and the food industry as a whole. As part of the Aflatoxin Elimination Workshop, presentations were made in the Crop Management and Handling, Insect Control and Plant Fungal Relations section that outlined the major crop management procedures, environmental variables, insect relationships and handling practices that were related to aflatoxin contamination in these commodities.

One major problem with aflatoxin contamination is extreme variability in the distribution of contamination and concomitant problems that the distribution imposes on sampling. This point was emphasized in the presentation on pistachios, but applies to all commodities.

In pistachios, early splitting of the hull and shell prematurely exposes kernels to insects such as the navel orangeworm and to fungi. As a result of fungal invasion and insect damage, early splits and shrivelled splits have discolored hulls, low kernel moisture, small size (both length and width) and/or low kernel weights, characteristics that can be used to remove these categories in color/size/weight separation procedures during processing. In addition, early splits have a characteristic staining along the hull suture. Current color sorting cannot distinguish the staining along the suture and removes many nuts that are not contaminated. Advanced current technology can use a combination of these characteristics for separating contaminated pistachios. For instance, near infrared radiation is used to separate dates and pecans in various categories according to kernel moisture levels, a practice that could be used to separate early split pistachios, since early split pistachios have characteristically low moisture.

Aflatoxin contamination in cottonseed may occasionally occur in each of the regions where cotton is grown. However, contamination occurs both more frequently and more severely in the irrigated desert valleys of west Arizona, south California, and south Texas. Populations of Aspergillus flavus increase during cotton production and spore distribution is partially homogenized by harvest and ginning. Differences in both the quality of A. flavus group propagules on the crop and the proportion of toxigenic A. flavus strains can be detected among different cotton growing areas. Pink bollworm damage, environment, and harvest practice are

important variables in determining the severity of aflatoxin contamination of cottonseed. Pink bollworm damage, environment, and harvest conditions were also discussed as important variables in contamination of cottonseed with aflatoxin. Transgenic cotton with the gene for the delta endotoxin from Bacillus thuringiensis offers potential for reduced aflatoxin contamination in cottonseed via control of the pink bollworm.

Important variables associated with aflatoxin contamination of peanut include drought for at least 21 days during the latter portion of the growing season and soil temperatures in the geocarposphere of 29-31çC. Soil insects such as the lesser cornstalk borer enhance infection of pods and kernels by feeding externally on the pod surface and/or penetrating the pod to feed on the kernel. Both types of insect damage result in higher levels of infection and aflatoxin contamination than is found in undamaged pods. Management variables that directly affect aflatoxin contamination in peanut are planting date, soil insect control, distribution of rainfall, irrigation, harvest date and harvest conditions.

Infection of corn with A. flavus may occur through the silk channel or via insect holes in the silk channel or through the husk. The major portion of kernel infection occurs through the pericarp rather than through the placental tissue. A high percentage of cobs may also become infected with A. flavus, but this infection is not necessarily related to kernel infection. Infection and contamination can be affected by planting date, variety, heat stress, insect damage and control, and other management practices. However, the most critical factor in the South/Southeast appears to be high evapo-transpiration stress during the last 30 days of seed maturation. Irrigation in south Texas during the last 30 days of seed maturation had little effect on reducing severe heat stress associated with increased infection in 1991. Insect resistance in the silk per se did not reduce aflatoxin contamination, but a tight husk cover over the ear tip seems to directly reduce aflatoxin contamination in kernels regardless of the susceptibility to insect damage.

In the Midwest, sap beetles are important vectors for the contamination of corn kernels with A. flavus. These insects commonly feed on fungi that invade damage sites made by other insects in corn stalks and ears and disseminate A. flavus propagules while feeding on fungi growing on the damaged tissue. Several techniques have been developed to monitor populations of these sap beetles and/or bait and control them as vectors. An integrated approach stressing cross resistance to insects and fungi, desirable corn characteristics, and vector control offers potential for management of aflatoxin contamination of corn in the Midwest.

Overall, the discussion of aflatoxin contamination in the various commodities revealed several areas of commonality, i.e. heat stress during seed maturation that resulted in high evaporative demand, insect damage during critical growth stages that enhanced exposure of developing fruit to A. flavus, and production variables that can be managed to reduce aflatoxin contamination.

AFLATOXIN ELIMINATION WORKSHOP

Crop Management and Handling, Insect Control and Plant Fungal Relations Section

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Relationships among strains in the *Aspergillus flavus* group which differ in toxin production, morphology, and vegetative compatibility group.

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The *Aspergillus flavus* group is composed of diverse asexual fungal strains which differ both morphologically and physiologically. Strain diversity has resulted in an array of taxa, many of which have limited use. More commonly recognized species are the aflatoxin producers, *A. flavus*, *A. parasiticus*, and *A. nomius*, and the non-aflatoxin producing species *A. tamarii*, *A. oryzae*, and *A. sojae*. *A. oryzae* and *A. sojae* have been used in food processing for over ten centuries and have been considered domesticated forms of *A. flavus* and *A. parasiticus*, respectively. Certain strains of both *A. flavus* and *A. parasiticus* also do not produce alflatoxins and may be used to prevent aflatoxin contamination through competitive exclusion. The group is further complicated by division of *A. flavus* into two formal strains, Strain S and Strain L, on the basis of both aflatoxin producing ability and sclerotial morphology. Phyletic relationships among members of this group are not clear; understanding these relationships may have a profound impact on our strategies to select safe and competitive atoxigenic strains to prevent aflatoxin contamination and on our understanding of the population biology of this group as a whole.

To differentiate *A. flavus* group isolates, strains, and species, a region of the Taka-amylase gene of each strain was amplified from genomic DNA with the Polymerase Chain Reaction (PCR) and variability within this region was examined by analysis of Restriction Fragment Length Polymorphisms (RFLPs). Primers (20 mer) were developed for a 1160 bp region of the Taka-amylase gene of *A. oryzae* from a published sequence (Tada et al. Agric. Biol. Chem. 53:593-599, 1989). A second set of primers, nested within the first primer set, was developed from the same sequence data. Amplification of *A. oryzae* genomic DNA with the first primer set resulted in a product of the size predicted from the sequence data. To confirm this amplification product was from the Taka-amylase gene, the second primer set was used to amplify the product of the first amplification. This second amplification also yielded a product of the size predicted from the sequence data, thus confirming that the amplification product was from the Taka-amylase gene.

The Taka-amylase gene provided better resolution of subgroups within the *A. flavus* group than did characterizations of mitochondrial rDNA also performed in our lab. When the initial 1160 bp Taka-amylase product was restricted and compared between species, strains designated *A. flavus* and *A. oryzae* differed considerably from strains designated *A. parasiticus* and *A. sojae*. Strains of both *A. flavus* and *A. oryzae* were very similar to each other, as were strains of both *A. parasiticus* and *A. sojae*. Toxin producers formed three distinct groups which correspond to *A. flavus, A. parasiticus*, and *A. nomius*. Relationships among many strains of *A. flavus* were not resolved. Other genomic regions are being investigated for that purpose.

Taka-amylase plays a critical role in nutrient capture in plant related ecological niches. Optimization of this enzyme's activity has also been a focus of industrial selection of strains of the *A. flavus* group. Detailed understanding of the evolution and differentiation of Taka-amylase among strains of the *A. flavus* group may also provide insight into both the population biology and evolution of this important group of fungi.

Aspergillus flavus: Wild Intruder or Domesticated Freeloader.

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Populations of Aspergillus flavus shift both qualitatively and quantitatively. During favorable environmental conditions, A. flavus populations may rapidly increase to over 10,000 propagules per gram of soil or crop matter. These populations also can rapidly decline when conditions no longer favor A. flavus, sometimes to levels below detection. Our field tests to date suggest that the initial A. flavus strains colonizing a crop during favorable conditions have the ability to compete with subsequently arriving strains and reduce their use of the majority of the crop's resources. There is apparently a great diversity of A. flavus strains adapted to growth on crop debris and infection of drought stressed crop plants. These vary from strains which produce large quantities of aflatoxins to strains which produce little or no toxins. Furthermore, the strains vary in other traits such as production of various enzymes and sensitivity to antifungal agents; some of these characteristics might be associated with increased safety of strains to humans and domestic animals. Application of select strains during the early stages of crop cultivation provides the opportunity to improve the safety of fungal populations associated with crops and agricultural products.

Through three years of field trials we have demonstrated that the aflatoxin producing ability of the fungal population associated with and infecting cotton crops can be reduced through application of atoxigenic strains. This reduction in toxigenicity is associated with a proportional reduction in the aflatoxin content of the crop. The choice provided by this technology is not whether or not fungi will be associated with our crops but rather whether we will select which fungi are associated through incremental domestication instead of allowing chance to determine the safety of these fungal populations.

We have performed 6 field experiments over this period. In all tests application of an atoxigenic strain during May or June influenced neither the quantity of A. flavus on the crop at maturity nor the percent of the crop infected by A. flavus prior to boll maturity. However, application increased incidence of the applied strains and decreased the aflatoxin content of the crop. In these studies three distinct atoxigenic vegetative compatibility groups have been shown to be effective. A long term population modification study from September 1988 till present indicates that A. flavus populations can rapidly turnover qualitatively. Strain infection of the crop is associated with subsequent overwintering of applied strains in the field soil. However, movement of strains from soil to the crop in subsequent years occurs in competition with strains moving into the field from surrounding areas. Strain ingress is probably enhanced by irrigation of test fields after planting and production of dust during cultivation of nearby fields. The results suggest that long term population modification will require treatment of relatively large areas.

Title of the project

Elimination of aflatoxins in Arizona using beneficial bacteria

Investigators

I.J. Misaghi and P.J. Cotty

To search for a bacterium capable of reducing aflatoxin contamination of cottonseeds, we screened over 800 bacterial isolates recovered from field soils, leaves, stems, and immature as well as opened bolls. Only six isolates showed partial or complete antagonistic activity against Aspergillus flavus in a cottonseed bioassay method developed in our laboratory. All six active isolates were originated from either immature or open bolls. One effective isolate (D1) was tested extensively in both greenhouse and field for the ability to prevent boll infection by A. flavus and to survive under field conditions.

A test plot was established in Yuma, Arizona during the 1992 season. Plants and soil under the canopy were sprayed with D1 8, 10, 14, and 17 weeks after planting. Leaf, boll and soil samples were collected periodically and were examined for the presence of the bacterium. The bacterium maintained higher population levels on bolls of field-grown plants than on leaves. It also multiplied rapidly on immature fibers of intact bolls in the field. Although D1 did not survive in dry field soil, it did reduce the level of A, flavus propagules in the soil early in the season. This activity may have been encouraged by flood irrigation. No visible signs of pink bollworm infestation or A. flavus-induced damage to bolls were observed in treated and control plots at the time of commercial harvest. Little pink bollworm damage occurred in the Yuma valley in 1992 and thus little boll rot was detected. All bolls collected from treated and control plants were fully fluffed, fibers were not stained and did not fluoresce under uv light.

Preliminary studies indicate that D1 can live both epiphytically and endophytically. The isolate D1, originally recovered from cotton bolls, seems to prefer cotton boll surfaces as an ecological niche. D1 can multiply rapidly on immature cotton fibers, live endophytically in plants, and can inhibit the growth of A. flavus in cotton bolls. These attributes make D1 well suited to serve as a biocontrol agent for A. flavus.

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## Biological Control

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Recent consumer concerns related to pesticide residues in the food supply require that alternative methods of pest control be developed. The use of biological control provides an attractive alternative to the use of pesticides. Since Aspergillus flavus and A. parasiticus do not require production of aflatoxin to invade plant tissues, some recent experimental studies on the use of non-toxigenic strains of A. parasiticus have shown some promise for preharvest aflatoxin management in peanuts.

The biocompetitive agent approach has been under investigation at the National Peanut Research Laboratory since CY 1986. We have achieved "proof of concept" and are in the process of refining this technology. We now know that we will need to develop a combination of A. flavus and A. parasiticus to be commercially acceptable. We also are required to use color mutants to facilitate soil analysis. We are currently investigating various color mutants developed via UV mutagenesis and are working with Dr. Linz's laboratories to develop genetically engineered mutants for biocontrol. In addition, we are evaluating the best form of the biocompetitive agent to use for effective biocontrol. Studies last year strongly suggested that sclerotial application was the best approach if you consider invasion of peanuts as the criterion as opposed to soil population density. We are also changing our philosophy on the relative importance of A. parasiticus vs A. flavus in the contamination of peanuts. A. flavus appears to be much more important than originally thought. Progress is slow since we are limited to conducting studies during the growing season.

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# Effect of Soil Populations of *Aspergillus flavus* and *A. parasiticus* on Infection of Peanuts

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Populations of *Aspergillus* species were monitored in a peanut and corn field in south Georgia. During this first year of study, populations of the *A. flavus* group in soil remained about 1500 cfu/g in both fields and comprised *A. flavus* and *A. parasiticus* in nearly equal proportions, with some indication of an increase in *A. parasiticus*. Of the 13 species of Aspergilli in the soil, only *A. flavus, A. parasiticus, A. niger, A. tamarii, and A. terreus* regularly invaded peanuts.

The role of *A. parasiticus* in infection of peanuts was examined more closely using environmental control plots in which drought and elevated soil temperature were induced to promote aflatoxin contamination during the latter part of the growing season. Soil of plots was inoculated with low and high levels of an orange-colored *A. parasiticus* (NRRL 6111) in the form of conidia, sclerotia, or mycelium (colonizing peanut seed particles). Peanut particles released abundant conidia into the soil, whereas sclerotia produced very few conidiogenous structures either in the field or in moist chambers. As peanuts matured, wild-type *A. parasiticus* in the uninoculated control plot increased to soil populations levels of *A. flavus*. In contrast, *A. flavus* dominated wild-type *A. parasiticus* (ratio 4:1) in all plots treated with the orange *A. parasiticus*. Infection of peanuts by *A. flavus* differed little between untreated and treated plots in most peanut maturity classes, whereas infection of all maturity classes by wild-type *A. parasiticus* was clearly reduced in plots treated with orange *A. parasiticus*. All maturity classes of peanuts from the high sclerotium plot showed a disproportionately high degree of infection by the orange *A. parasiticus* compared to the other treatments. Differences in soil populations of the orange *A. parasiticus* were correlated with the frequency of infection in peanuts.

The orange *A. parasiticus* preferentially excluded wild-type *A. parasiticus*, but not *A. flavus*, from peanuts. *A. flavus* and *A. parasiticus*, though closely related, may therefore have different, yet still largely unknown, ecological niches. Application of nonaflatoxin-producing isolates to competitively control natural aflatoxigenic populations might require a combination of both species for peanuts.

**Evaluation of Selected Geocarposphere Bacteria as Biological Control Candidates Against *Aspergillus flavus* Colonization of Peanut**

by C.J. Mickler, K.L. Bowen and J.W. Kloepper

Bacteria found in the geocarposphere (the zone around the subterranean pod) of peanut has been found to be distinct from the bacteria found in the peanut rhizosphere (root zone). Indigenous geocarposphere strains, since they are ideally suited for colonizing developing pods, could make ideal biocontrol agents against aflatoxigenic fungal invasion of peanut seed. In 1991, geocarposphere bacteria were collected and screened for biocontrol potential. Nineteen of these strains were selected for their ability to inhibit *Aspergillus flavus* colonization of peanut seed and root radicles in laboratory trials. A preliminary greenhouse has been conducted with several of the 19 best strains. Candidate bacteria were inoculated onto peanut seed planted in 5 gal pots. Two weeks after planting, soil in pots was infested with *A. flavus* contaminated corn kernels. Peanut plants were grown to maturity. All bacterial strains observed in this study reduced geocarposphere populations of *A. flavus* and pod infection compared to a control with no bacterial treatment. Bacterial strains were observed to differ in their ability to reduce geocarposphere populations and/or pod invasion by *A. flavus*.

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Preharvest Control of Aflatoxin
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AFLATOXIN WORKSHOP

OCTOBER 31-NOVEMBER 3, 1992

FRESNO, CALIFORNIA

# WASTE CORN AS AN INOCULUM SOURCE FOR ASPERGILLUS FLAVUS

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## ABSTRACT

Visible sporulation of _A_. _flavus_ was detected on deposits of waste corn at all 16/17 cribs and 4/5 storage bins examined in 1991 and 1992. Airborne inoculum of _A_. _flavus_ was detected at 9/14 cribs and at 4/5 bins tested. These values were as much as 20 times greater than typical spore count obtained in corn fields. The data indicated that there was extensive _A_. _flavus_ inoculum on waste corn and in the air at cribs and bins throughout Iowa, and that the degree of infestation of the fungus varied among sites.

In order to determine if inoculum of _A_. _flavus_ on waste corn can be transmitted by air and nitidulid beetles to neighboring corn fields, an intensive study was made in 1992 at two cribs surrounded by waste corn at Cedar Rapids and Williamsburg, Iowa. Airborne spores of _A_. _flavus_ were measured and nitidulid beetles were trapped at different times and at various distances from the deposit of waste corn into an adjacent corn field. At the same time measurements were made of _A_. _flavus_-infection of leaves and silks on corn plants at different distances from the source. A second experiment was carried out in replicated field plots at Ames, IA in which piles of waste corn were brought in from a naturally contaminated site and placed in the middle of plots. Corresponding non-infested plots were paired with infested plots and each plot was replicated four times. Measurements were made of airborne spores, insects and infection of leaves and silk at different times during the season.

At each of the three locations, a distinct gradation in spore density and plant infection in relation to distance from the waste corn was established. These data provided strong evidence that waste corn was an inoculum source of _A_. _flavus_ and that the fungus is dispersed in the air to infect corn plants.

Insect trapping showed much higher nitidulid beetle counts occurred at 2-6 m from waste corn deposits than at further distances. Substantial percentages of these insects were infested with _A_. _flavus_, The data indicated that waste corn was a major source of _A_. _flavus_-contaminated nitidulid beetles.

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Aflatoxin Elimination Workshop
Fresno, California, November 1-3, 1992

# MYCOPARASITES AND CHEMICAL DEFENSES IN SURVIVAL OF ASPERGILLUS SCLEROTIA

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Our research program is organized with the goal of integrated pest management of Aspergillus flavus and aflatoxin. One of our objectives is to investigate factors limiting the survival or germination of A. flavus sclerotia in both irrigated and non-irrigated corn field soils. We are examining the biocontrol potential of sclerotial mycoparasites (i.e. Paecilomyces lilacinus; Trichoderma pseudokoningii). This research also investigates the sclerotia of A. flavus and closely related species for novel antiinsectan metabolites that protect the sclerotia from fungus-feeding insects that cohabit soil and crop debris. The starting point for this collaboration was the key observation that the sclerotia of A. flavus are avoided by the common detritivorous beetle Carpophilus hemipterus, an insect that feeds on the conidia and mycelia of the same fungus (Wicklow et al., 1988. Trans. Brit. Mycol. Soc. 91: 433-438.). Dihydroxyaflavinine was isolated and found to be a potent antiinsectan sclerotial metabolite when incorporated into a pinto bean diet at 100 p.p.m. D.W. Naturally occurring levels of this metabolite are higher in A. flavus sclerotia. Dihydroxyaflavinine proved non-toxic to vertebrates at 300 mg/kg. These findings led us to initiate general studies of the chemistry of Aspergillus sclerotia as sources of new antiinsectan natural products, including several novel compounds with oral activity against Helicoverpa zea comparable to that of the commercial synthetic insecticides malathion (an organodithiophosphate) and permethrin (a pyrethroid). Results of our bioassay guided studies, 1987 to date, have led to the discovery of over seventy natural products, forty five (45) of which possess previously unreported chemical structures including eight (8) new ring systems. These new antiinsectan sclerotial chemicals are of particular interest to the fields of agriculture and medicine for their biological activities and potential commercial application(s) and are presently being tested by several U.S. companies.

Investigations of antiinsectan Aspergillus sclerotial metabolites are in collaboration with P.F. Dowd, Mycotoxin Research Unit, NCAUR, Peoria, and J.B. Gloer, Department of Chemistry, Univ. of Iowa. The research is supported by a grant from the National Science Foundation and through a Cooperative Research and Development Agreement No. 58-5114-0-4003 with the Biotechnology Research and Development Corporation, Peoria, Illinois.

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# Construction of a DNA Probe for Distinguishing Vegetative Compatibility Groups in *Aspergillus flavus Link:Fr.*

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## ABSTRACT

Repetitive DNA sequences have proven useful and reliable characters in evaluating genetic relatedness of strains at different levels of classification. A DNA probe was constructed to determine the genetic identity of *Aspergillus flavus* isolates using DNA fingerprinting techniques. Chromosomal DNA of *A. flavus* (NRRL 6541) was digested with EcoRI and ligated to Lambda Dash bacteriophage. Four potential probes were identified in Lambda clones and all four were subcloned into pUC19 by transforming competent *Escherichia coli* TG1 cells. Plasmid DNA from all four clones were labeled with digoxigenin, using a random primer labeling technique and a chemiluminescent detection system. Southern blots of total DNA revealed that all four cloned DNA probes hybridized to multiple restriction fragments, suggesting that the cloned sequences recognize repetitive DNA in EcoRI, PstI or HindIII digests of *A. flavus* DNA. However, only one (p28L2) of the four subcloned DNA probes could distinguish all 21 vegetative compatibility groups (VCG's) among 28 strains as characterized by K. E. Papa (Mycologia 78:98-101). This DNA probe may be species-specific as it hybridized with all isolates of *A. flavus* we tested, with strains that some recognize as varieties of *A. flavus* (e.g. *A. oryzae, A. parasiticus*, and *A. sojae*) but not with the DNA of other fungal species tested (e.g. *A. alliaceus, A. melleus, A. nomius, A. ochraceus, Fusarium moniliforme,* and *Penicillium thomii*). Since *A. flavus* was first discovered in preharvest corn, there has been a continuing controversy about the origin of infective inoculum, the importance of airborne fungal spores versus corn insect vectors. The probe we developed should enable us to distinguish genetically distinct population of *A. flavus* within the same corn field. We are presently contrasting *A. flavus* populations isolated from the corn at harvest in 1989, 1990, and 1991 with the *A. flavus* populations isolated from air, soil, crop residues, and corn insects as the corn developed in the field those same years.

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# Ecology and Biological Control of Aflatoxigenic Fungi in Tree Nut Orchards in California

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**Ecology.** From pistachio orchards in California the following fungi in the *Aspergillus flavus* group have been isolated: the most common, *A. flavus* strain L (large sclerotia), *A. flavus* strain S (small sclerotia), *A. flavus* strain O (no sclerotia), *A. parasiticus*, and *A. tamarii*. *A. flavus* strain O has several characteristics (colony color, growth rate under certain conditions, etc.) distinct from the other strains. Testing the isolates for aflatoxin production in media showed that only 36% of strain L isolates, 93% of strain S isolates, 100% of strain O isolates, and 100% of *A. parasiticus* produced both B and G aflatoxins.

Similar to our 1991 results, *A. flavus* was associated with pistachio debris on the orchard floor. In five commercial pistachio orchards, 26% of male inflorescences and 0.8% of the surface-sterilized pistachio fruits on the ground developed *A. flavus/parasiticus* after incubation. The availability of pistachio debris for colonization by *A. flavus* from late spring throughout summer would increase *A. flavus* levels in pistachio orchards and provide spore inoculum for infections of kernels mid to late summer.

**Cultural practices.** Substantial reductions in the amount of irrigation only slightly reduced the number of early splits (abnormal nuts with split hulls and shells that are frequently contaminated with aflatoxins). In one experiment, reducing irrigation 75% slightly reduced the percentage of early splits from 1.4 to 1.0%. In another experiment, withholding irrigation for two months before harvest did decrease early splits by 62%, but drastically decreased yields. Withholding irrigation until after mid May resulted in 138% increases in early splits. The percentage of early splits for an orchard increased as the percentage of normal nuts with split shells increased. Nuts with *A. flavus/parasiticus* were found in orchards representing all of the common cultural practice and there was no clear indication that any specific cultural practice favored infection of nuts by *A. flavus*.

**Biological control.** Two approaches were used for biological control of *A. flavus* using antagonistic microorganisms. The first approach was to protect nuts on the tree (i.e., early splits) from infection by *A. flavus*. In 1992, we improved our method for evaluating potential antagonists in research orchards. Two biocontrol agents (*Penicillium* sp. and *Aspergillus tamarii*) were successful in decreasing *A. flavus* growth in pistachio kernels. The second approach was to apply the antagonists to debris on the orchard floor in order to inhibit development of *A. flavus* on these debris. Approximately 150 new potential antagonists were isolated. Fifty-four of these were tested for effectiveness as biocontrol agents on pistachio male inflorescences and fruits in the lab. Nineteen percent of the antagonists completely inhibited *A. flavus* sporulation on male inflorescences. Nine percent of the test antagonists decreased *A. flavus* sporulation on pistachio fruits by more than 95%. So, we now have several promising biocontrol agents available for preliminary field testing.

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# Fungal Flora of Pistachios from Harvest Through Processing

**A. Douglas King Jr., Melanie Flores, Susan Rodriguez and Noreen Mahoney**

Aflatoxin has been reported to be a rare but recurring event in pistachio nuts. The aim of this research was to examine the mycology of pistachio nuts to detemine how the fungal population relates to aflatoxin in the nuts. We defined the fungal population, especially *Aspergillus flavus,* of pistachio kernels and examined competition between molds on the kernels. Pistachio nuts were sampled at points after harvest through processing and storage. The nuts were examined for fungal flora by planting kernels on Dichloran Rose Bengal Chloramphenicol Agar (DRBC) and Dichloran-18%Glycerol Agar (DG18). The same two media were used to determine fungal counts. Nine species of molds were commonly isolated from kernels. Fungal counts increased with harvest date but the counts from different harvest dates were similar after drying. *Penicillium* spp. dramatically increased with harvest date. *Aspergillus niger* was present on all samples as the most prominent mold. *Rhizopus stolonifer* increased after hull removal and decreased during drying. *Penicillium* spp. also decreased during processing and drying. *Aspergillus flavus* was present on samples in-hull and during processing. The percentage of kernels with *A. flavus* increased after drying and into storage. Approximately 30 percent of *A. flavus* cultures were toxigenic.

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# AFLATOXIN PRODUCTION IN PISTACHIOS

S.B. Rodriguez, N.E. Mahoney, D.W. Irving, A. D. King, Jr.

Pistachios picked from trees near Madera, California on September 22, 1992 and from an orchard north of Sacramento on October 9, 1992 were transported to our laboratory for inoculation with a toxigenic strain of *Aspergillus flavus*. Pistachios were sorted into nuts with intact hulls and "early-split" nuts. Those with intact hulls were separated into mature and less mature, "greener" hulls. A cut was made on hulls of one third of the nuts with intact hulls. These cut nuts were sorted into nuts with split and unsplit shells. Another third of the nuts with intact hulls were dehulled.

Nuts were inoculated with 7, 100 or 200 *A. flavus* spores. Nuts with intact hulls were inoculated on the hull surface. Nuts with hull cuts were inoculated in the cut. Dehulled nuts were inoculated through the split shell, directly on the kernel. "Early-split" nuts were inoculated in the split. Some of the cut-hull nuts were reserved to inoculate at varying times, up to 42 hours. After a 4-day incubation at 30$^{o}$C, pistachios were assayed for aflatoxin. Experiments were done in triplicate, with 12 nuts per sample. Hulls and kernels + shells were assayed separately. In two additional cut-hull samples, nuts were assayed individually.

Aflatoxin was not detected in hulls or in any nuts with unsplit shells. Nuts inoculated in hull cuts with 7 or 200 spores reached similar levels of aflatoxin in 4 days (6000 ppb). Nuts with "greener" hulls contained more aflatoxin (15,800 ppb) than nuts with mature hulls (6,300 ppb). Dehulled nuts inoculated with 200 spores contained twice the amount of aflatoxin as cut-hull nuts; however, dehulled nuts inoculated with 7 spores contained only 40% (2,500 ppb) of the aflatoxin found in nuts with cut hulls in 4 days. "Early-split" nuts contained less aflatoxin than any other group of nuts (840 ppb). Nuts inoculated more than 30 hours after hulls were cut resulted in reduced *A. flavus* infection, increased *A. niger* infection and decreased aflatoxin content (92% decrease in 42-hr samples). Although 21 of the 22 cut-hull nuts assayed individually had visible *A. flavus* infection, only 8 contained aflatoxin: 50; 200; 220; 300; 23,400; 32,200; 50,800, 87,000 ppb AFB1. The aflatoxin-contaminated nuts could not be differentiated from the aflatoxin-free nuts visually or by fluorescence. The variability in aflatoxin level per kernel is discussed.

A concentration of 60,000 ppb aflatoxin in one nut is equivalent to a 20 ppb average in a 10-lb lot of pistachios containing that nut. The following model for the production of > 60,000 ppb aflatoxin in one pistachio nut is suggested:

(1) A toxigenic *A. flavus* spore lands in a recent (< 30-hour) wound. It germinates and grows in the wound site, establishing its dominance in relationship to the predominant mold, *A. niger*. No aflatoxin is produced during this phase.

(2) Hyphae grow into the kernel and synthesize > 60,000 ppb aflatoxin within 4 days of infection if conditions (e.g. chemical composition of hull and kernel, $CO_2$ level in kernel) are optimum.

# Summary: Panel Discussion on Opportunities to Manipulate Microbial Ecology

The Panel consisted of : M. Doster, B. Horn, D. King, D. McGee, and I. Misaghi. The discussion was chaired by P. Cotty who also prepared this summary. Comments were also made by R. Cole, P. Dowd, C. Holbrook, J. Robens, and N. Zummo, as well as others.

After a summary of the talks, the floor was opened for discussion which is paraphrased below.

Could modification of cultural practices reduce contamination of the various crops? King pointed out that the populations of fungi associated with nut crops he and his colleagues have studied reflect an infection potential. Handling of the crop will dictate the success of that potential. Doster suggested that modification could effect vulnerability of pistachio crops by modifying percent early splits but, he suggested the practicality of this may be questionable. McGee pointed out that his results suggest very simple management procedures to eliminate inoculum foci in the midwest and thus delay A. flavus population increases. McGee felt these cultural modifications were well within what midwest growers might be willing to implement.

Horn was questioned about the implications of his observations on displacement of native A. parasiticus during peanut infection by an orange mutant of A. parasiticus but failure of this strain to displace native A. flavus strains. He suggested that the results might indicate different relationships between these two fungal species and the peanut plant; the fungi might even infect either by different mechanisms or through different infection courts.

Holbrook commented that differences in infection by A. flavus and A. parasiticus might impact on his project to breed for resistance in peanuts. Some of the breeding is being done in Arizona where A. parasiticus may be less common than in Georgia. Cotty commented that there will probably be multiple genes governing resistance and that some may be active against only one or the other fungus. Some may be active against all aflatoxin producers.

Misaghi suggested natural distribution of potential biocontrol organisms may influence the distribution and severity of contamination. Misaghi emphasized that biocontrol is operating in agricultural fields and that the development of a biocontrol organism often seeks to increase the incidence and intensity of an activity already present, rather than to create a new activity.

Cole questioned the importance and distribution of A. parasiticus on the various crops. Doster indicated that A. parasiticus occurs on tree crops but not so frequently as A. flavus; Zummo indicated that A. parasiticus was effective at rotting corn. Cotty said that A. parasiticus could also affect cotton bolls. They said A. parasiticus occurred at a low frequency on corn and cotton at the locations in which their research was undertaken. It was pointed out that failure of Cotty to find A. parasiticus in his Arizona samplings may indicate either low sensitivity of his detection method or a prejudice in his sampling rather than the absence of A. parasiticus. Cotty sampled primarily cotton

fields. Horn indicated that he is looking into the relative contributions of A. parasiticus and A. flavus to contamination of peanuts.

Michailides asked if A. tamarii had potential as a biocompetitive agent. Cotty indicated that A. tamarii did inhibit aflatoxin contamination of cotton bolls when coinoculated with A. flavus but, he did not feel it would be adequately competitive with A. flavus under field conditions favoring contamination because, based on A. tamarii's distribution, it is apparently adapted to a different set of environmental conditions. Cotty, Doster, and Michailides indicated that A. tamarii occurs on cotton and tree crops, but generally at lower rates than A. flavus. Cole commented that A. tamarii had potential for contaminating crops with cyclopiazonic acid (CPA) since most A. tamarii isolates seem to produce CPA.

Dowd questioned the safety of applying biocompetive fungi to agricultural fields. Cotty indicated that he felt safety concerns would be adequately addressed because biocompetition did not result in greater overall populations of fungi but, rather fungal populations with unchanged population levels and improved safety characteristics. Cotty suggested that normal agricultural practice applies large quantities of debris colonized with fungi to fields annually and that agricultural workers are exposed to extremely high fungal populations on a regular basis. Cotty also suggested that safety concerns might be directed not only at reducing the aflatoxin producing potential of fungal populations but also at other characteristics of fungi considered deleterious to humans or human activities.
Cotty emphasized his results showing fungal populations could be modified dramatically by timely application of specific strains and suggested that caution should be taken in field experiments using toxigenic strains to apply only indigenous strains.

Robens commented that a similar biocompetitive approach is being taken in the poultry industry where young birds are being seeded with appropriate microflora capable of excluding organisms (i.e. Salmonella) detrimental to humans.

The importance of Aspergillus inoculum in dust generated during standard agronomic practice was discussed as it relates to transport of the fungus to cotton bolls and to other crops. Michailides commented that his lab had shown a direct linear relationship between amounts of soil dust accumulated on fig trees and incidence of fig smut caused by Aspergillus niger; a curvilinear relationship between propagules of A. niger on fig leaves and fig smut also exists. Michailides suggested that something similar may occur for A. flavus and A. parasiticus in pistachio orchards but, it is very difficult to show such relationships because the incidence of pistachio infection by A. flavus and A. parasiticus is very low.

# AFLATOXIN BIOSYNTHESIS: MOLECULAR ELUCIDATION AND INHIBITION OF THE PATHWAY

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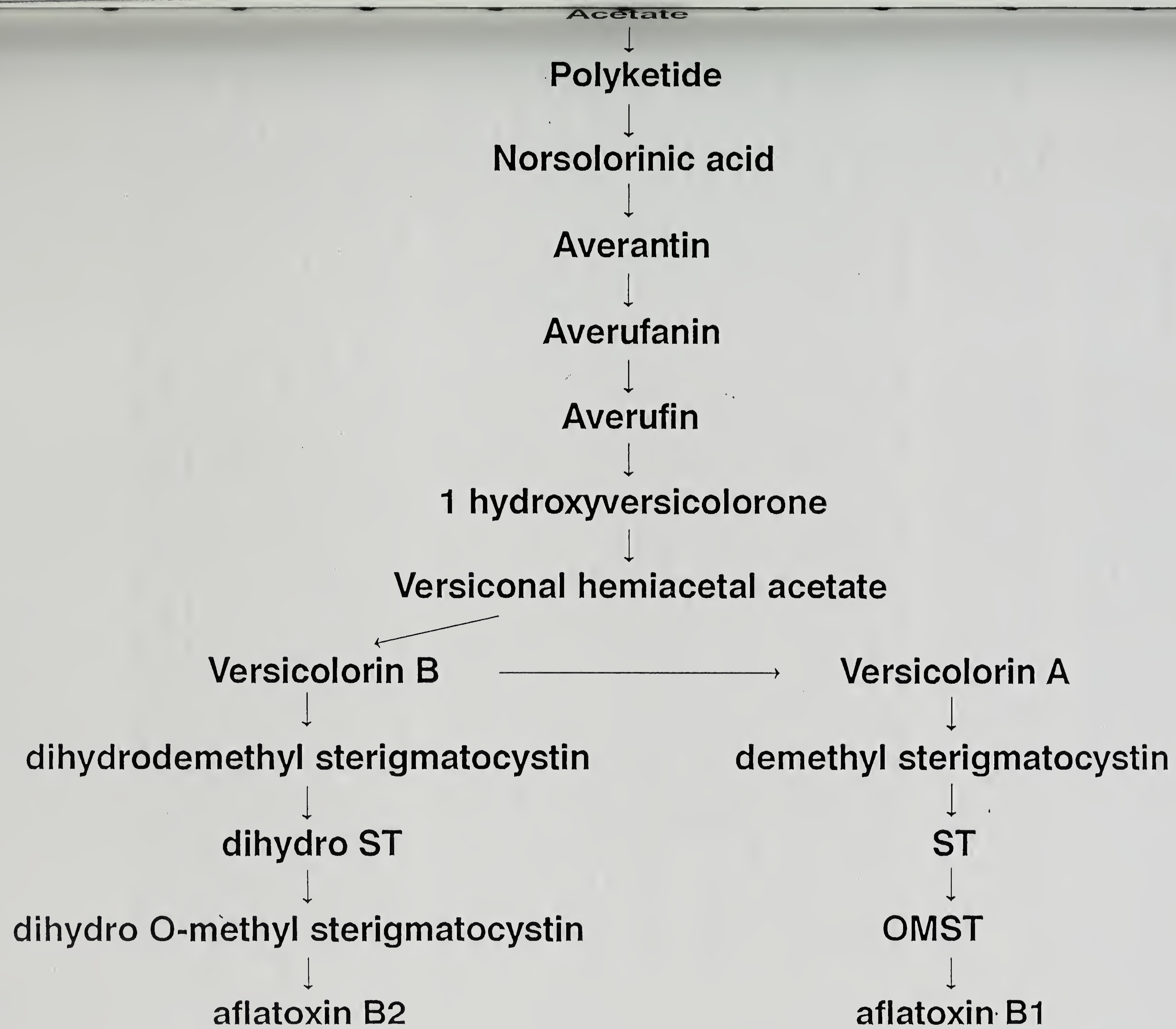
Aflatoxin synthesis has no obvious physiological role in primary growth and metabolism of the organism and, therefore, is considered to be a "secondary" process. As yet, there is no confirmed biological role of aflatoxin in the ecological survival of the fungal organism. However, since aflatoxins are toxic to certain potential competitor microbes in the ecosystem, a survival benefit to the producing fungi is implied. It should be noted, however, that aflatoxin per se is a poor antibiotic. It has been proposed that intensive agricultural practices are responsible for the creation of unique niches that under certain conditions select toxin-producing fungi. Contemporary crop production is based on intensive practices, and it is unlikely that these practices will be altered in a significant manner in the near future. It is, therefore, imperative to develop a comprehensive understanding of both the mycology of aflatoxin contamination in various ecosystems and the molecular regulation of aflatoxin formation. This understanding may ultimately provide the tools for developing strategies for effective control of aflatoxigenic fungi and elimination of aflatoxin contamination from animal feed and human food chains.

Several chemical and biochemical factors involved in aflatoxin formation by the fungi Aspergillus flavus and A. parasiticus have been elucidated earlier (Figure). Recent observations from this lab include the following: (1) versicolorin A is the precursor of aflatoxin B1 and versicolorin B, an aflatoxin $B_2$ precursor, is the progenitor of versicolorin A. The A. flavus $B_2$-accumulating mutant is blocked in the ver B --> ver A conversion step; this strain is being used to study genetics of the branch in pathways of $AFB_1$ and $B_2$ synthesis; (2) Oligonucleotide and antiserum probes, synthesized based on the purified and characterized enzymes (methyltransferase and norsolorinic acid reductase) are being utilized to identify the genes coding for these aflatoxin pathway enzymes; (3) Utilizing fungal transformation strategies, two genomic DNA fragments have been cloned that result in opposite phenotypes in transformed strains with regard to aflatoxin biosynthesis. In these studies Dr. Linz' NA cosmid (containing the nor-1 and ver-1 genes) was utilized. A 4.5 kilobase fragment of genomic DNA from the cosmid inhibited aflatoxin production in an aflatoxigenic A. parasiticus strain, without affecting any enzyme activity in the pathway. Another 2 kilobase fragment of DNA from the same cosmid caused overproduction of all aflatoxin precursors in a producing fungal strain. These factors, that putatively regulate aflatoxin biosynthesis, appear to be linked to the genes, nor-1 and ver-1, involved in aflatoxin synthesis; (4) Based on these observations, a model for the regulation of aflatoxin pathway genes has also been postulated involving signals from plants turning on certain regulatory genes in fungi and resulting in the expression of aflatoxin pathway genes and subsequently aflatoxins.


Acetate
Polyketide
Norsolorinic acid
Averantin
Averufanin
Averufin
1 hydroxyversicolorone
Versiconal hemiacetal acetate
Versicolorin B
Versicolorin A
dihydrodemethyl sterigmatocystin
demethyl sterigmatocystin
dihydro ST
ST
dihydro O-methyl sterigmatocystin
OMST
aflatoxin B2
aflatoxin B1

# CHARACTERIZATION OF GENES FOR AFLATOXIN BIOSYNTHESIS

Gary A. Payne, North Carolina State University,
Deepak Bhatnagar and T.E. Cleveland, USDA/ARS/SRRC

Aflatoxin biosynthesis is under regulatory control within *Aspergillus flavus* and *A. parasiticus*. We have isolated a gene from *A. flavus* that is involved in this regulation. This gene, *afl-2*, regulates aflatoxin biosynthesis before the formation of the first stable intermediate, norsolorinic acid. It also regulates other enzymatic steps in the pathway. Thus, *afl-2* appears to have a pivotal role in aflatoxin biosynthesis. Because of the importance of this gene, we have focused our research efforts on the characterization and the role of *afl-2* in aflatoxin biosynthesis.

We have shown that *afl-2* resides within a 1.8 kb piece of DNA. The gene appears to be transcribed at a low level, as we have not been able to detect a clear band for the transcript by Northern analyses. We have, however, identified a clone in our cDNA library that hybridizes to this gene. The cDNA is 1029 bp in length and has an open reading frame of 702 bp. This open reading frame codes for a putative peptide of 234 amino acids and has a molecular weight of 26,113 daltons. A comparison of the cDNA and peptide sequences of *afl-2* with database sequences did not reveal significant homology; therefore, this gene appears to be different from previously described genes. We also used *afl-2* to probe a Southern blot of DNA from *A. parasiticus*, *A. nidulans*, *Cercospora kikuchii*, and *Cochliobolus heterostrophus*. The probe hybridized only to *A. parasiticus* DNA, indicating that *A. parasiticus* contains a comparable gene to *afl-2*. These results suggest that information derived from our research on *afl-2* will have implications in *A. parasiticus* as well as *A. flavus*.

In order to determine more about the genomic organization of aflatoxin biosynthesis and the position of the *afl-2* gene in relation to other pathway genes, we have begun studies to locate *afl-2* to chromosome. We have resolved 7 of the 8 chromosomes of *A. flavus* by CHEF electrophoresis and we are in the process of assigning marker genes to each of these chromosomes. We are fortunate in that 30 genes have been mapped to linkage group in *A. flavus*, including 11 genes for aflatoxin biosynthesis. Our objective is to isolate marker genes for each of the 8 linkage groups and to use these genes as probes to identify the corresponding chromosomes. To isolate linkage group marker genes, auxotrophic strains were transformed with a genomic library and complementing clones were recovered. To date, 7 marker gene clones have been recovered: *thi-1 arg-2*, *lys-4*, *arom-4*, *pdx-6*, leu-7, and *arg-7*. We have used three of these marker genes to identify the chromosomes of linkage groups 4, 6, and 7. We also have shown that *afl-2* does not resides on one of these three chromosomes.

We have begun studies to characterize the promoter region of *afl-2* to determine the elements of the promoter that are important for transcription. To do these studies we developed a GUS reporter system that allows us to measure promoter activity by the production of a colored product. We have constructed a β-glucuronidase vector (pGAP4) that permits the ligation of promoter sequences into a polylinker. To test the utility of this construct we created a vector containing this construct fused with the constitutive promoter of the *A. flavus* β-tubulin gene and the inducible promoter of the *A. flavus* alcohol dehydrogenase (*adh-1*) gene. These vectors were introduced into an aflatoxin producing-strain of *A. flavus* (WWI) and examined under conditions conducive and non-conducive for aflatoxin formation. Both promoters drove the β-glucuronidase gene in *A. flavus*. Expression of GUS activity driven by the β-tubulin gene was similar for both media whereas expression by the inducible *adh-1* construct was 8 fold greater on inducing medium than noninducing medium. The expression of GUS had no apparent effect on fungal growth or on aflatoxin production. These results provide the foundation to exploit a GUS-reporter gene system for studying promoters of genes involved in aflatoxin biosynthesis.

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# IMMUNOCHEMICAL STUDIES ON THE ENZYMES OF AFLATOXIN BIOSYNTHESIS

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Attempts to produce both monoclonal (Mab) and polyclonal (Pab) antibodies against 3 key enzymes involved in the aflatoxin (AF) biosynthesis, i.e. sterigmatocystin (ST) methyltransferase (ST-MTFase), norsolorinic acid reductase (NSR), and polyketide synthetase (PKS), were made. The objectives for these studies are: (1) to use immunoaffinity method for purification of the enzymes, (2) to further our understanding on the structure and function of the these enzymes, (3) immunochemical neutralization of the enzymes, and (4) to clone genes of these enzymes in collaboration with USDA scientists by supplying them with immunochemical reagents for enzyme characterization. Progress of our work during the past two years is summarized as follows:

## A. Production and characterization of Mab and Pab against norsolorinic acid reductase (NSR):

Norsolorinic acid reductase (NSR), which catalyzes the conversion of norsolorinic acid (NSA) to averantin (AVT), is one of the key enzymes involved in the early stage of aflatoxin biosynthesis. With the supply of the partially purified NSR from Dr. Bhatnagar of Southern Regional Research Center (SRRC) of USDA, we have successfully obtained both monoclonal (Mab) and polyclonal (Pab) antibodies against the enzyme. An indirect ELISA was established for measurement of the antibody titer using partially purified NSR as a coating antigen and a second antibody-peroxidase conjugate as an indicator. Polyclonal antibodies against NSR were demonstrated in rabbits 10 weeks after the animals were immunized with a purified enzyme preparation (2 bands). Western blot revealed that the antibodies reacted with two major proteins of 48kd and 38 kd size. The partially purified antisera (ammonium sulfate ppt) were able to inhibit the enzyme activity associated with both proteins. Indirect ELISA revealed that the antibodies reacted with culture extracts from almost all the AF producing fungi tested. *Aspergillus sojae* and *Penicillium* spp. didn't react with the antibody. However, there was some cross-reaction of the antiserum with *Fusarium* spp.

Further separation of the 48kd and 38 kd proteins was achieved by Sephadex G-100 gel filtration. The 38 kd protein was then conjugated to Sepharose gel, which was then used as an affinity column for the separation of antibodies specific for the 48 kd and 38 kd proteins. Further analysis revealed that antibodies against the 48 kd protein inhibited enzyme activity. The enzyme preparation containing primarily the 48 kd protein was selected to immunize BABL/c mice for production of monoclonal antibodies. A total of 12 hybridoma cell

lines that produced monoclonal antibodies against various proteins were obtained. Analysis of the partially purified Mab (ammonium sulfate ppt) by the Western blot analysis showed that different clones produce antibodies with diverse specificities. Immunochromatography of a crude *A. flavus* culture extract revealed that NSR activities located in various HPLC fractions were associated with the immunoreactivity when the Mab produced by clone 1D9 was used in the ELISA. This Mab primarily reacted with the 48 kd protein band as well as a minor band of 43 kd size protein. In contrast, the Mab produced by clone 10D2 reacted with primarily the 43 kd protein with a minor reaction with the 48 kd proteins.

Considerable efforts were made to determine which of the protein bands (43 kd vs 48 kd) were NSR. Earlier data from USDA and our laboratories indicated that NSR activity was found to be predominately associated with the 48 kd protein species. However, recent results obtained from the immunoaffinity column absorption technique, immuno-neutralization and Western blotting studies showed that the NSR activity was associated with the 43 kd protein species, and the antibody produced by hybridoma cell line 10D2 is specific for the NSR. Attempts to re-clone 10D2 cell line were made and 7 new hybridoma cell lines were obtained. Nevertheless, antibodies produced by these clones have the same characteristics as those from 10D2 clone.

Two other studies also supported the conclusion that Mab produced by 10D2 is specific for NSR: (1) Mab obtained from 1D9 clone reacted with the protein band at 48 kd size in the extract of both toxic and non-toxic *Aspergillus, i.e. A. parasiticus* 2043 (AFB1 producer) and 284 (non-producer), *A. oryzae* 1191 and *A. parasiticus* mutants including AVR, 163 AVN-1, and VER in the Western blot. In contrast, Mab from 10D2 clone, reacted only with the 43 kd protein band from those extracts showing positive for NSR activities (*A. parasiticus* 2043, AVN-1, VER, and AVR), (2) kinetics analysis for the formation of NSR and AFB production by *A. parasiticus* 13007 (AFB producer) revealed that NSR activity and AFB production were closely related to the formation of 43 kd protein bands as detected by the Western blot and ELISA when the 10D2 Mab was used.

A small amount of both Pab and Mab against NSR was sent to the scientists in SRRC of USDA for their work in cloning of the gene that encodes the enzyme. Both antibodies have been shown to be good probes for the identification of NSR genes.

<u>B. Production and characterization of Mab and Pab against sterigmatocystin (ST) methyltransferase (ST-MTFase):</u> The second enzyme selected in the present study is sterigmatocystin (ST) methyltransferase (ST-MTFase), an enzyme involved in the conversion of ST to aflatoxin. Investigations at SRRC have led to the isolation and purification of two types of ST-MTFase with molecular weights of about 168 and 40 kd, respectively. Polyclonal antibodies against the large molecular weight ST-MTFase have been produced and characterized by the USDA scientists. Using the Pab against the high molecular weight enzyme, an indirect ELISA that can detect

ST-MTFase at levels between 0.2 to 2.0 ug/mL (10 to 100 ng protein/assay) was established in our laboratory. Indirect ELISA of various fungal extracts revealed that most of the non-aflatoxin fungi, including species of *Aspergillus, Penicillium* and *Fusarium*, did not cross-react with the antibody. However, extracts obtained from some non-aflatoxin producing aspergilli such as *A. sojae* and *A. oryzae* had 5-10% cross-reactivity with the antibodies in this assay system.

With the supply of a semi-purified enzyme preparation by SRRC scientists (3 bands on SDS PAGE), polyclonal antibodies against the low molecular weight enzyme preparation were recently obtained in our laboratory. Western blot revealed that the antibodies reacted primarily with one major and two minor protein bands of 40-45 kd size. Analysis of the crude enzyme preparation obtained from *A. parasiticus* strain 163 revealed only one band corresponding the ST-MTFase in the Western blot (c.a. 40 kd). However, several other protein bands that didn't show any enzymatic activity were also reacted with the antibodies when high concentrations of the antibodies were used in the assays. The antibodies were subsequently purified by passing the antiserum through an affinity column that was prepared by conjugating the proteins with no-enzyme activity (isolated by DEAE-cellulose chromatography). This antiserum has been used successfully in the cloning studies by the USDA scientists.

Production of monoclonal antibodies against ST-MTFase was also initiated using the same enzyme preparation as the immunogen. A total of 14 hybridoma cell lines shown to elicit antibodies against the crude enzyme preparation were obtained. Studies for further characterizations of both Pab and Mab are presently underway.

<u>C. Production of antibodies and development of immunoassay for polyketide synthetase</u>. Investigation in Dr. Linz's laboratory at Michigan State University has led to the identification of the genomic DNA sequence that may be involved in the biosynthesis of polyketide (PKS), one of the earliest events in the biosynthesis of AF. Part of the sequence of the 682 bp ClaI/HindIII DNA fragment that encodes AF-PKS shows strong similarity with the sequence that encodes patulin PKS. However, attempts to isolate the enzymes at USDA were unsuccessful primarily because the lack of specific substrate and product for these enzymes. With the availability of the DNA sequence, two immunogens containing that part of polypeptide sequences were prepared. One immunogen contained a polypeptide fragment having an amino sequence that is completely identical both in the AF-PKS and patulin-PKS; the other, however, is partially identical. An indirect ELISA, similar to the one for monitoring of Mab for NSR, was used for antibody titer determination. Antibody against these two peptides were demonstrated six weeks after immunization when the original immunogens were coated on the ELISA. However, the antibody titers were low when *A. flavus* crude culture extracts were coated on the ELISA plate. Further characterization of the antibodies and their interaction with PKS will be carried out. We hope to use these antibodies for isolation of AF-PKS from *A. flavus/A. parasiticus*.

# Complementation of a Mutation in the Aflatoxin Biosynthetic Pathway of Aspergillus parasiticus prior to Norsolorinic acid.

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**Abstract**

The aflatoxins are a group of secondary metabolites that are produced by certain strains of closely related imperfect fungi Aspergillus parasiticus and A. flavus and are the most potent naturally occurring carcinogens known. Elimination of aflatoxins from the food chain requires a fundamental understanding of their biosynthetic pathway. The long term goal of this research project is to eliminate aflatoxins from food and feed at the preharvest level. The short term goal is to develop genetically stable nontoxigenic strains of Aspergillus parasiticus which can be safely utilized in the field to reduce or eliminate aflatoxin contamination of food or feed crops by biological exclusion.

In past research we have cloned 2 genes, nor-1 and ver-1, associated with aflatoxin biosynthesis. We recently have developed constructs which should simultaneously allow us to measure nor-1 and ver-1 promoter function (reporter gene fusions) and to disrupt the chromosomal gene copy. These disruption strains will be useful in detecting the effects of disruption on aflatoxin biosynthesis, growth and development. However, because of their position in the biosynthetic pathway, disruption of the nor-1 and ver-1 genes will not generate useful biocontrol strains because they will still produce low levels of aflatoxin (nor-1)or versicolorin A (ver-1) which are toxic compounds.

For this reason we focused on isolating a gene at the earliest possible step in the pathway. We have generated two UV mutants (UVM7 and UVM8) blocked in an early step (prior to norsolorinic acid). These mutants were derived from Aspergillus parasiticus strain B62 (niaD, nor-1, br-1) which accumulates norsolorinic acid. Metabolite conversion studies with major known intermediates of the aflatoxin biosynthetic pathway suggest that the block in the pathway in both mutants occurs prior to norsolorinic acid. Feeding the whole cells with norsolorinic acid showed that a very small fraction of norsolorinic acid could be converted to aflatoxin indicating that UVM7 and UVM8 have double blocks, one of them being before and the other at norsolorinic acid. We have been able to complement UVM7 and UVM8 mutants to aflatoxin production with the cosmids nor-A and nor-B in two separate experiments. One of the UVM7 transformants accumulated norsolorinic acid, suggesting that only the mutation prior to nor-1 was complemented. Preliminary restriction endonuclease mapping data of nor-A and nor-B cosmids suggest that the genes that complement mutations in UVM7 and UVM8 are confined to a ~21 kb DNA fragment. Our future research will concentrate on subcloning and characterization of the structure, expression, and function of the gene(s) responsible for complementing UVM7 and UVM8 phenotype. This will be followed by construction of disruption vectors for the UVM7 and UVM8 gene(s).

"Isolation of an *Aspergillus nidulans verA* gene homologue"

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Abstract

Sterigmatocystin (ST) and aflatoxins (AF) are similarly structured polyketide mycotoxins produced by *Aspergillus* spp. such as *A. flavus, A. nidulans, A. parasiticus, A. ustus* and *A. versicolor.* Most of the AF pathway - up to ST - is found in several *Aspergillus* species including the genetically well characterized *A. nidulans.* A scheme of the pathway intermediates leading to $AFB_1$ is: polyketide precursor > norsolorinic acid > averantin > averufanin > averufin > versiconal hemiacetal acetate > versicolorin A > sterigmatocystin > O-methylsterigmatocystin > $AFB_1$. We have identified and partially characterized an *A. nidulans* gene, *verA*, proposed to encode a reductase active in the ST/AF biosynthetic pathway. The putative product of this gene shares extended homology to the *A. parasiticus ver-1* gene and partial homology to several polyketide biosynthetic genes involved in polyketide production in *Streptomyces* spp. The *A. nidulans* gene is located on pL24B3, a cosmid which also contains a chromosomal region with homology to a putative polyketide synthase. It is possible that pL24B3 contains a gene cluster involved in AF/ST biosynthesis.

Experimental antecedents suggest that molecular studies of *A. nidulans* will increase the speed in which the scientific community can understand the regulation of the AF pathway. *A. nidulans* offers several experimental advantages over *A. parasiticus* and *A. flavus* for examining the molecular genetic mechanisms controlling AF/ST production including (1) its characterized DNA transformation system, (2) establishment of advanced molecular studies of regulation of *A. nidulans* genes and (3) its history as a genetic model with both a sexual and asexual stage. For these reasons, *A. nidulans* was successfully used as a model system to study penicillin production. In addition, because AF production has been related to developmental changes in strains of *A. flavus* and *A. parasiticus* , the availability of well characterized *A. nidulans* developmental mutants may be useful for studying regulation of secondary metabolite production. Current projects include sequencing and characterizing the regulatory (promoter) region of *A. nidulans verA*, determination if the *A. nidulans verA* gene will compliment the AF mutation in *A. parasiticus* strain CS10, and a *verA* gene knock out in an *A. nidulans* ST producing strain for proof of function.

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**Selected Publications:**

Keller N P, Adams T H (in preparation) Isolation of an *Aspergillus nidulans verA* Homologue on a Putative Sterigmatocystin/Aflatoxin Gene Cluster. Curr Genet

Keller N P, Owen J, Bhatnagar D, Cleveland T E (1992) Utility of PCR for Diagnosis of *Aspergillus* Species. APS 1992 Annual Meeting, Phytopathology, abstr.

Keller N P, Dischinger Jr H C, Bhatnagar D, Cleveland T E, Ullah A H J (submitted) Purification of a 40 KDa Methyltransferase Active in the Aflatoxin Biosynthetic Pathway. Appl Environ Micro

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# Genetic Regulation of Aflatoxin Biosynthesis

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Abstract

Two mutagenesis strategies are employed to detect genes that contribute to the genetic regulation of aflatoxin. One scheme utilizes an *A. flavus* strain, which is constitutive for aflatoxin production, so all aflatoxin genes are functioning. The regulatory system in this strain is faulty allowing aflatoxin to form continuously beginning at early log phase. Mutagenesis of this strain yielded ten aflatoxin regulatory and several pathway intermediate mutants. We are currently analyzing these mutants. A second scheme utilizes several *A. flavus* strains repressed for aflatoxin and all aflatoxin biochemical intermediates. One of these strains was mutated to high aflatoxin production. When this regulatory mutant was combined with the repressed parent, aflatoxin biosynthesis was completely repressed. Repression in the diploid, however, was found to be variable depending on the growth media. When grown and tested on a peanut medium, the repressed diploid produced aflatoxin. These results demonstrate the importance of testing all presumptive aflatoxin regulatory mutants on peanut medium.

We have recently isolated four mutants from a UV irradiated *A. parasiticus* NOR mutant; these mutants do not accumulate any colored anthraquinones or aflatoxin on peanut medium. We are currently preparing these genetic variants for analysis in diploid to determine dominance /recessive relations with regard to aflatoxin regulation.

In an earlier study we carried out a differential screening procedure with a genomic DNA library from *A. parasiticus* with three different cDNA probes related and unrelated to aflatoxin biosynthesis. These experiments generated 19 genomic clones positively correlated with and presumably enriched with genes associated with aflatoxin production. We have selected four of these clones for sequencing. Restriction maps for each clone have been determined, sequencing has been carried out and we are presently searching a gene bank for information regarding relatedness of these sequences to DNA sequences with known functions.

PANEL DISCUSSION SUMMARY:

## MOLECULAR BIOLOGY OF A. FLAVUS AND AFLATOXIN FORMATION

Panel Members: Thomas E. Cleveland, Deepak Bhatnagar, Fun Sun Chu, and Gary Payne, Chair

Three approaches are being used by the Biosynthesis Group to elucidate the aflatoxin pathway. The first approach is enzyme isolation. Enzymes for aflatoxin biosynthesis are present in low quantities, many are unstable, substrates are often insoluble, and the assay procedures are cumbersome. For these reasons, little was known about the mechanistic details of the pathway when this research group was formed. The use of molecular techniques along with specific antibodies has enable Drs. Bhatnagar, Chu, Cleveland, and Keller, to isolate three enzymes in the pathway.

The second approach being used to study aflatoxin biosynthesis is gene isolation by complementation for function. When this research began no genes in the pathway had been isolated, and some scientists were skeptical that such a strategy would work for a secondary metabolite such as aflatoxin. Using this strategy, however, two genes (nor, ver) have been isolated from A. parasiticus by Dr. Linz and a regulatory gene (afl-2) has been isolated from A. flavus by Dr. Payne. The ver and nor genes reside on the same cosmid and Dr. Linz has evidence that other pathway genes are clustered around these two genes. Further, Drs. Bhatnager and Payne have shown that A. parasiticus has a homologue of the afl-2 gene and that it resides in the same gene cluster as nor and ver. In contrast, Dr. Payne has shown that in A. flavus the afl-2 does not reside on the same chromosome as nor, indicating that the genomic organization of the pathway in the two fungi differ.

The third approach to gene isolation is by complementary DNA hybridization. Dr. Leonard has isolated several cDNAs for transcripts induced during aflatoxin formation. Drs. Bhatnagar, Cary and Payne have sequenced and characterized three genes by a similar approach. The role of these genes in the pathway is unknown.

The results of the Biosynthesis Group show that the approaches being used will allow us to isolate genes and enzymes in the biosynthetic pathway and study their regulation. We attribute the success of this group to the hard work of its members and the spirit of cooperation that exists among the scientists within the group. The multiple approaches and cooperation among individuals have lead to advances in understanding the pathway beyond what we imagined when we began.

What can we do with the information gained from these studies? Ultimately, we hope to use the information to devise a strategy to disrupt the biosynthetic pathway. A long term goal is to engineer plants to produce compounds that inhibit aflatoxin biosynthesis. An immediate use of the findings is for the development a gene-specific assay to screen plant genotypes. Such an assay would be based on the ability of plant exacts to inhibit or induce promoter activity of one of the pathway genes. Promoter activity can be easily measured by fusing the promoter to a reporter gene

such as GUS. The expression of GUS activity can be measured by the production of an easily visualized compound. The advantages of such an assay are 1) it would be easier and safer than aflatoxin analyses, 2) it would be more sensitive and less variable because it directly measures a gene in the pathway, and 3) it would allow the identification of compounds that block the early steps in the pathway. It is important to block the early steps in the pathway as intermediates in the pathway are also toxic.

We think that the disruption of aflatoxin biosynthesis as a strategy for aflatoxin elimination is a sound approach. Evidence is accumulating that compounds exists in crop plants that inhibit aflatoxin biosynthesis. Such a mechanism may exist in plants that do not accumulate aflatoxin. This strategy along with a strategy to confer resistance to the fungus could lead to the elimination of aflatoxin accumulation in plants.

# DEVELOPMENT OF A TRANSFORMATION SYSTEM FOR PEANUT

A. K. Weissinger[1], P. Ozias-Akins [2] and T. E. Cleveland[3]

Our laboratories have evaluated two different systems by which alien genes could be introduced into peanut. In the first procedure, DNA is delivered by microprojectile bombardment of embryogenic callus derived from immature embryos. These cultures are capable of continuous embryogenesis when maintained on media containing the growth regulator picloram, and regenerate sexually functional plants when transferred to medium without picloram. Numerous transformed culture lines and transformed plants have been recovered by this route, from somatic embryos to which a chimeric hygromycin phosphotransferase (HPH) had been delivered. Bombarded tissue was grown on non-selective media for four weeks, and then transferred to selective media containing hygromycin. Either solidified medium with 10 mg/L hygromycin or liquid medium with 20 mg/L hygromycin were used. Although stably transformed tissue was recovered from both selection protocols, selection in liquid media permitted more rapid elimination of non-transformed tissue. Both somatic embryos and plants recovered from bombarded tissues under selection were shown to carry the HPH gene by PCR amplification and by Southern blot analysis for presence of the HPH sequence.

In an unrelated procedure, alien DNA is delivered by microprojectile bombardment of leaflets from peanut seedlings. Plants can be regenerated from this tissue through direct organogenesis. Selection to recover transgenics was carried out with kanamycin. While this antibiotic unequivocally selected transformed callus from bombarded tissues, plants surviving selection were all found to have escaped selection. No true transformants were recovered. This system is now being reevaluated using methotrexate as the selective agent.

We are now working to produce transgenic peanut germplasm with enhanced resistance to *Aspergillus flavus.* In a series of pilot experiments, numerous embryogenic callus cultures have been bombarded with a chimeric chitinase gene obtained from Dr. Richard Broglie of DuPont de Nemours & Company, Inc. This genetic construct has been shown to protect transgenic tobacco against infection by *Rihzoctonia* spp., but has not yet been tested against *Aspergillus.* The chitinase bearing plasmid was delivered in combination with a plasmid carrying the selectable HPH marker gene. Both runner and large-seeded Virginia type peanut cultures have been treated. These cultures are now under hygromycin selection, and are expected to yield transgenics cotransformed with both the HPH and chitinase genes.

Efforts continue to improve the transformation system itself, and to produce transgenic germplasm with enhanced resistance to *Aspergillus.* We are continuing to improve the gene transfer system, using stable transformation efficiency, rather than transient expression, as an indicator of efficiency. We are also opening new collaborations with other workers who could supply additional antifungal genes, which will be introduced into the transformation system as they become available.

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# IDENTIFICATION OF ASPERGILLUS FLAVUS GROWTH AND AFLATOXIN BIOSYNTHESIS INHIBITORS: SOURCES OF RESISTANCE GENES FOR GENETIC ENGINEERING OF CROPS

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Fungal growth and aflatoxin biosynthesis inhibitors are being characterized to determine the feasibility of cloning the genes encoding these inhibitors and using these genes to genetically engineer crops for resistance to infection by Aspergillus flavus/parasiticus and aflatoxin contamination. An inhibitor of aflatoxin biosynthesis, synthesized in cottonseed coats during a transient period of resistance of the seed to aflatoxin contamination, was further characterized for potential identification of the gene(s) encoding the inhibitor(s). An inhibitor of aflatoxin biosynthesis in corn kernels associated with the living kernel embryo was detected and is being further characterized for the cloning of the gene(s) involved.

Cell wall degrading enzymes produced by A. flavus during invasion of cotton bolls and corn kernels were identified; pectinases, cellulases, xylanases, proteinases and amylases all were detected during early stages of fungal infection suggesting a role of these enzymes in infection and spread of the fungus through plant tissues. Plant genes have been cloned which encode proteinaceous inhibitors of several of these fungal enzymes (e.g., pectinases, proteinases and amylases) produced during fungal infection; therefore, inhibitor genes (e.g., the proteinase inhibitor II gene) are being used to genetically engineer cotton and to enhance resistance to invasion by A. flavus.

Peptide analogs based on the structure of the lytic peptide, cecropin, were ineffective in inhibiting growth of A. flavus. An oxyradical-generating human myeloperoxidase system was highly inhibitory to growth of A. flavus suggesting that the gene for this enzyme could be useful in genetic engineering plants for fungal resistance.

In the search for potent antifungal enzymes and associated genes for use in plant genetic engineering, over one hundred isolates of chitinolytic bacteria were screened for chitinase producing ability and growth inhibition of A. flavus and A. parasiticus. Most bacterial isolates were highly chitinolytic on chitin amended agar medium, but only three isolates demonstrated ability to inhibit growth of both species of aflatoxigenic fungi in zones between fungal and bacterial colonies. This finding suggests that some chitinases (or other hydrolases) produced by the bacteria may possess high specificity for inhibition of a particular fungal strain; this further suggests that in the initial screening of microbial hydrolase genes for use in genetic engineering of plants, genes should be selected which encode hydrolases with specificity against a broad spectrum of fungi. Chitinase genes from selected bacterial strains will be used in the genetic engineering of cotton, peanut (stable transformation and regeneration of peanut has recently been accomplished; see "Summaries" by Ozias-Akins and Weissinger) and other crops subject to aflatoxin contamination.

# STABLE TRANSFORMATION OF PEANUT AND REGENERATION OF TRANSGENIC PEANUT PLANTS

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The focus of the transformation research over the past funding period has been to select for stably transformed tissues of *Arachis hypogaea* and to regenerate transgenic plants from these tissues.

Somatic embryos and embryogenic callus can be induced in vitro from immature zygotic embryo explants of peanut. Induction and maintenance occurs on a nutrient medium supplemented with the synthetic auxin, picloram. Long-term embryogenic cultures of selected genotypes of *Arachis hypogaea* have been maintained for 12-24 months. A culture protocol that circumvents an embryo maturation step has been imposed to encourage shoot development from apical meristem regions of somatic embryos. Embryogenic callus pieces approximately 25 $mm^2$ containing an estimated 1-5 somatic embryos were cultured on media with either NAA or a combination of cytokinins. An average of 5-6 shoots per callus piece can be produced. Subsequent transfer to basal medium and medium supplemented with gibberellic acid allowed the formation of elongated shoots which were subsequently rooted. Rooted shoots transferred to the greenhouse flower and produce pegs.

Long-term, regenerable embryogenic cultures were bombarded with DNA-coated microprojectiles. Plasmid DNA contained a hygromycin-resistance gene (*hph*) driven by the CaMV 35S promoter. Bombarded callus was grown for one subculture period (about one month) under non-selective conditions and subsequently was transferred to liquid medium containing 20 mg/l hygromycin. After approximately two months, hygromycin-resistant calli were recovered. Fourteen callus lines have shown integration of the *hph* gene based on amplification of the gene by the polymerase chain reaction. Seven of the callus lines were tested for integration of the foreign gene by hybridization of the gene to genomic DNA from the transgenic lines. Regenerated shoots from two callus lines have all shown the presence of the *hph* gene based on amplification by the polymerase chain reaction. All shoots tested by Southern analysis have shown the presence of integrated foreign DNA.

**Publication**

Ozias-Akins, P. W.F. Anderson, and C.C. Holbrook. 1992. Somatic embryogenesis in *Arachis hypogaea* L.: Genotype comparison. Plant Science 83:103-111.

Title: Genetic Engineering of Cotton to Enhance Fungal Resistance

Authors: Caryl A. Chlan and Rachel O. LaPorte (Biology Department, The University of Southwestern Louisiana, Lafayette, LA 70504) and Jeffrey Cary, and Thomas Cleveland (Southern Regional Research Laboratory, USDA/ARS, New Orleans, LA 70179)

We are in the process of developing the technology to transform and regenerate cotton after introducing genes for antifungal proteins. Cottonseed is an economically important byproduct of the cotton industry, and its cash value is affected by levels of aflatoxin contamination. Conventional control measures are ineffective, and naturally resistant varieties are not available. Genetic engineering of cotton is a feasible, logical approach to generate cotton with an increased resistance to Aspergillus flavus. Transgenic cotton can be obtained by Agrobacterium Ti vector transformation, followed by regeneration of the transformed tissue into mature plants. This technology has been successfully applied to cotton, and transgenic insect resistant cotton plants have already been field tested.

The specific objective of this project is to generate transgenic variants of cotton that resist invasion and infection by Aspergillus flavus. In order to achieve this goal, we first need to prepare genetically engineered strains of Agrobacterium that carry fungal resistance genes under control of promoters that will yield either wound induced, seed specific, or constitutive expression. These Agrobacterium strains will be used to transform cotton tissue, from which transgenic cotton plants regenerated. The transformed plants will then be scored for the stable expression of genes, and their resistance to fungal infection and growth. Resistant plants will be propagated.

Currently, we have developed three test Agrobacterium strains. One contains a constitutive promoter (Cauliflower mosaic virus 35S) driving the expression of a reporter gene Beta-glucuronidase, the second contains a cottonseed storage protein promoter driving the expression of Beta-glucuronidase, and the third contains the CaMV 35S promoter attached to a proteinase inhibitor gene.

The Agrobacterium strains mentioned above have been used to transform cotton tissue derived from Coker 312. We have propagated an isolate of Coker 312 5A that is more easily transformed (supplied by Dr. Norma Trolinder), and have at our disposal enough seed for the transformation and regeneration experiments. We have been concentrating on transforming and regenerating cotton hypocotyl tissue. We have treated sterile hypocotyl tissue sections (derived from freshly germinated surface sterilized seeds with all three of the above Agrobacterium strains, washed the tissue, and are currently maintaining it on selective media. Our initial experiments indicate that longer preculture times favor more callus formation on transformed hypocotyl sections. The process of cotton regeneration is slow; to date we have successfully regenerated transformed cotton cells to callus and suspension cell stages. Suspension cells have been plated onto embryogenic medium, and undifferentiated growth has been observed, however, no embryos have developed as yet.

Our immediate plans are to continue work on the regeneration of cotton plants from tissue culture. Towards this goal, we are regenerating non-transformed cotton tissue as well as transformed tissue. The non-transformed tissue is healthier, and grows faster, which provides a good system for rapid development of the tissue culture techniques needed for regeneration of cotton plants from hypocotyl sections. Currently we have non-transformed tissue that developed to the stage of embryo differentiation. We are also in the process of identifying additional potential antifungal genes to introduce into cotton via hypocotyl transformation and plant regeneration.

# PROGRESS IN INSERTION AND SCREENING OF CHITIN-BINDING GENES IN WALNUT

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In walnut, as in many tree nuts, aflatoxin contamination reduces marketability and is considered a serious problem. One approach to reducing contamination involves insertion of fungus growth inhibiting genes, such as the chitin-binding genes. Walnuts are a promising system for testing the feasibility of controlling *Aspergillus flavus* through genetic engineering because an efficient transformation system has been developed in our lab, and the resulting transgenic embryos can be used for rapid bioassays of gene activity. The chitin-binding genes encode proteins which bind glucans of glycoproteins. In fungi with chitin-glucan hyphal walls, binding of the lectins to the chitin chains at the tip of the emerging hyphal tubes has been shown to limit the extension process. Our goal is to insert chitin-binding genes in walnut and to screen transformed embryos for response to *A. flavus*. The system will be designed to be adaptable for insertion and screening of other genes with potential for controlling *A. flavus*.

Seven different gene constructs (barley lectin sense & antisense, hevein sense & antisense, *Urtica dioica* agglutinin (UDA) sense & antisense, and unmodified pMON893, all in *Agrobacterium tumefaciens* LBA4404 were used in experiments designed to transform 4 different walnut embryo lines. Standard walnut transformation techniques were used. A total of 10 embryo subclones are multiplying well on selective medium and will be further multiplied for Southern blot analysis and screening for response to *A. flavus*. All the potential transformants were recovered from the SU2 clone and none from the 3 other clones which have proven not to be very embryogenic, even as controls.

To increase selection efficiency, the gene encoding the ß-glucuronidase (GUS) scorable marker is being incorporated into the constructs. The vector plasmid pRTL2-GUS/SV was provided by A. Dandekar and the pMON 893 vector with the barley, hevein, and UDA sense constructs in *E. coli* MV1190 by T. Wilkins. The GUS fragment obtained by HindIII digest of pRTL2-GUS/SV was ligated to pMON 893 linearized with HindIII. The new vector construct designated pDU 92.16 was multiplied in *E. coli* DH5, before being inserted into *Agrobacterium* strain AGL1. Insertion has been accomplished in the hevein construct and work is still in progress for the barley and UDA constructs.

To develop a method to screen somatic embryos for reponse to *A flavus* a non-aflatoxin producing strain of A. *flavus* was used to inoculate mashed vs intact somatic embryos of clone SU2. Each treatment was replicated 3 times and consisted of 6 embryos or 6 x _90mg of mashed tissue placed in wells in 0.6% water agar. Inoculum, $10^3$ spores, was added to each well. Embryos were then incubated in the dark at 32°C and 80% RH. Three days after inoculation, 100% of the wells with mashed embryos were covered with *A. flavus* conidia, whereas only 5% of intact embryos showed fruiting. The rapid response obtained with wounded tissue may be useful for screening purposes.

# MOLECULAR ANALYSIS OF A PECTINASE GENE FROM ASPERGILLUS PARASITICUS

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Pectinases produced by Aspergillus flavus and A. parasiticus are believed to play a significant role in the ability of these fungi to spread in cotton bolls. A DNA probe was generated by PCR of a Aspergillus niger pectinase gene. The PCR probe was used to screen an A. parasiticus (SU1) cDNA library generated from mRNA isolated from glucose grown mycelia. The cDNA insert of the clone, designated PEC1, was aproximately 1.3 kb in length. DNA sequence analysis and the predicted amino acid sequence of PEC1 demonstrated a significant degree of homology at the amino acid level with pectinases of bacterial, fungal, and plant origin. Northern blot analysis of RNA isolated from SU1 grown on glucose or pectin as sole carbon sources showed that PEC1 was expressed during growth in both media. This finding indicates that PEC1 encodes a pectinase that is constitutively expressed and not subject to catabolite repression. A pectinase of this nature has been identified in culture filtrates of A. parasiticus and highly-virulent strains of A. flavus grown on glucose as a sole carbon source. The PEC1 gene will be used to isolate the analogous gene from A. flavus so that its regulation in both high and low-virulence strains can be studied. Gene inactivation studies can then be performed to determine the degree with which this pectinase plays a role in the ability of A. flavus and A. parasiticus to invade and spread in cotton bolls and other crops.

# UTILIZATION OF SPECIFIC BACTERIAL ANTIFUNGAL GENES FOR THE CONTROL OF AFLATOXIN CONTAMINATION

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Chitinases are antifungal enzymes which degrade fungal cell walls by hydrolyzing B-1,4-linkages of chitin. Chitinolytic bacteria, as sources of antifungal genes, were tested in vitro for their antagonistic effects against the aflatoxin-producing fungi Aspergillus flavus and A. parasiticus; and Fusarium moniliforme, a fungus which produced a wide range of toxins including fumonisins. One hundred bacteria were selected from the Auburn University chitinolyic bacterial collection for initial testing. Eight were chosen for further antifungal studies based on their high chitinase activity. Bacteria were grown in the presence and absence of chitin for three days prior to the introduction of fungi. Chitinase activity was noted by the development of a clearing zone in the chitin plants surrounding the bacterial colonies. Three of the bacteria tested showed no inhibition of any of the fungi despite high chitinase activity as noted by clearing zones. Serratia marcescens demonstrated species-specific inhibition of the three fungi as shown by inhibition of A. flavus, but did not inhibit A. parasiticus, and only partially inhibited F. moniliforme.

These results may indicate a specificity between chitinases produced by the different bacteria and their enzymatic properties, as well as antifungal activities against various fungi. A strain of Bacillus (AU192), identified as B. subtilis by GC-FAME analysis, had the greatest inhibition of all fungi tested both in the presence and absence of chitin. In assays the basal level of chitinase activity in the uninduced cultures was 15-20% of the induced chitinase activity. This indicates a constitutive as well as a regulated expression of chitinases which correlates with antifungal activity. Microscopic observations showed that the hyphae in the clearing zones were lysed. Genomic DNA of AU192 was isolated, digested with EcoRI, fractionated by agarose gel electrophoresis, and was probed with the radiolabelled chiA-D gene of B. circulans. Homology was seen between the B. circulans genes and specific EcoRI fragments of AU192. Isolated fragments will be cloned into expression vectors to determine chitinase activity. The ultimate goal of this project is to express these antifungal genes constitutively in transgenic plant tissues utilizing engineered plant expression vectors and determine resistance of transgenic plant tissues to toxin-producing fungi.

## Characterization of Aflatoxin-Metabolizing Enzymes from Aspergillus Spp.

E. H. Gendloff, W. E. Kenealy, and E. B. Smalley, University of Wisconsin, Madison.

Several studies have indicated that aflatoxin-producing aspergilli possess enzymes which metabolize aflatoxin. We wish to purify and characterize this activity.

**Our objectives** are to: **1.** Purify aflatoxin-metabolizing enzymes from soluble and microsomal fractions of fermentor-grown Aspergillus parasiticus. **2.** Determine the major products of the reaction of the enzymes with aflatoxin $B_1$ (AFB1) and compounds related to AFB1. Use this information to determine kinetics and probable enzymatic mechanisms of degradative reactions. **3.** Determine toxicity and mutagenicity of reaction products characterized under objective 2.

Previously, using ELISA to determine AFB1 concentrations, we found that some ammonium sulfate-precipitated extracts of shake culture-grown A. parasiticus NRRL 13007 mycelium could degrade AFB1 into non-immunoreactive products over several days. Various growth conditions, cofactors, and enzyme stabilizers were tested for their effect on enhancement of this degradative activity, with mixed results. Our findings to this point have shown us that the degradative activity present in the extracts we have prepared so far has not been sufficient to purify a degradative enzyme in an efficient manner.

In attempts to increase the degradative activity present in mycelial extracts, we have grown mycelium of aflatoxin-producing and nonproducing A. parasiticus in 12L SLS medium in a 16L fermentor. Large quantities of homogeneous stationary phase mycelium could be produced in this way, and large amounts of mycelial extract could be prepared. Using ELISA, we have screened subcellular, microsomal, and ammonium sulfate precipitated fractions from these mycelial extracts, as well as ammonium sulfate precipitations from culture filtrates for degradative activity. We have not found degradative activity from these fractions that was more rapid than the activity described with shake culture-grown mycelium.

We have recently abandoned ELISA for quantitation of AFB1 in these assays because we found that there was significant cross-reactivity of the ELISA with important oxidized forms of aflatoxin such as aflatoxin $Q_1$, aflatoxin $M_1$, and aflatoxin $B_{2a}$. We are therefore rescreening the various fractions described above for AFB1 degradative activity using TLC with fluorimetric quantitation. During this rescreening we have found that micromolar concentrations of sodium periodate are very effective in degrading AFB1 in a weak potassium phosphate buffer, but not in water. The single visible degradation product comigrates with aflatoxin $Q_1$. Work is continuing to positively identify the degradation product and to further characterize this reaction.

Other related work by our group involves the potential use of mammalian cytochromes P-450 to detoxify aflatoxin. Several P-450s will convert aflatoxin into less toxic or non-toxic forms. Some of these have been cloned. We hope to utilize some of these enzymes in transgenic crop plants in order to confer on those crops the ability to degrade aflatoxin into a nontoxic product. The genes for these enzymes might also be inserted into yeast used for ethanol fermentation so that aflatoxin contaminated corn could be decontaminated during the fermentation process, thus rendering the fermentation byproduct nontoxic for animal feed.

Gendloff, E. H., F. S. Chu, T. Leonard. 1991. Degradation of Aflatoxin by Lactoperoxidase and Mycelial Extracts of Aspergillus parasiticus. (Abstract). Phytopathology 81:1175.

Panel Discussion: "Future Obstacles to be Overcome in the Genetic Engineering Approach."

Panel members included Caryl Chlan, University of Southwestern Louisiana; Peggy Ozias-Akins, University of Georgia, Tifton; Gale McGranahan, University of California, Davis; Arthur Weissinger, North Carolina State University and Chairman, Ed Cleveland, Southern Regional Research Center, ARS, New Orleans, LA.

The discussion centered around the following technologies (suggested during the previous 1991 workshop) that have to be developed before a genetic engineering approach to incorporate resistance to aflatoxin contamination of crops can be accomplished:

1. Identification of inhibitors of A. flavus and A. parasiticus growth and/or aflatoxin biosynthesis; and

2. Identification of genes for fungus/aflatoxin inhibitors.

One of the main obstacles to genetic engineering plants for resistance to aflatoxin contamination was considered to be characterizing the inhibitors that are potent A. flavus growth and/or aflatoxin pathway inhibitors. Several potential fungal growth inhibitors (eg. chitinases) and genes encoding the inhibitors are available which inhibit plant pathogenic fungi, in general, but which either do not inhibit A. flavus or have not been tested in bioassays against A. flavus due to unavailability of purified inhibitor proteins. Activities inhibiting A. flavus growth and aflatoxin biosynthesis have been detected in various plants and microbes, but the inhibitors have not been chemically characterized or identified for future cloning of pertinent genes.

In addition to inhibitors of toxin synthesis per se, we should also consider alternative approaches including identification of plant metabolites which either induce toxin synthesis or participate in toxin metabolism. It might be possible to alter some aspects of seed (peanut kernels, etc.) composition which would reduce the "induction" effect or reduce rate of toxin synthesis by removing or reducing key metabolic intermediates. Finally, we might also consider transformation of plants with genes encoding enzymes which could alter aflatoxins once they are produced (in essence a detoxification strategy). This last approach has the advantage of allowing interaction between gene product of the plant and aflatoxin, rather than between gene product and the fungus.

Complete characterization of A. flavus growth and aflatoxin inhibitors remains a critical technological need for cloning of genes coding for these inhibitors for use in genetic engineering of plants. It was also suggested that genes for insect toxins (Bt toxin) which have already been cloned and stably expressed in plants should be included (in addition to fungal inhibitor genes) in the genetic engineering strategy. Insect vectors only indirectly contribute to entry into the plant by aflatoxigenic fungi, but probably result in a large percentage of the fungal infections leading to contamination by aflatoxin.

3. Stable transformation of crops with inhibitor genes.

The general consensus seemed to be that stable transformation has been demonstrated with a number of the commodities that we are interested in engineering. In particular, stable transformation of cotton and walnut with the Bt gene has been demonstrated and peanut has been transformed with a reporter gene and regenerated plants obtained. There was some discussion concerning which inhibitor genes would be most effective in reducing levels of aflatoxin in the targeted crops. In particular, in the case of peanut, would it be best to focus on introduction of the Bt gene, or are there other approaches that would be more promising? Because the process of plant transformation and subsequent regeneration are lengthy, the panel wanted to know if there were specific targets on which to focus. The consensus seemed to be that a multifaceted approach would be the best way to approach the problem of reducing aflatoxin contamination. Antifungal and insecticidal products have been shown to have effect in transgenic plants in previous studies. Our best strategy at present is to acquire genes known to function in plants and test them in vitro (if possible) for activity against A. flavus. Those with activity against A. flavus could be introduced into plant systems and tested against the fungus in plants. This approach is needed because of the large number of genes available. We cannot afford to transform crops with all of them.

No genes have yet been isolated with demonstrated activity against aflatoxin synthesis or for detoxification of aflatoxins. It is likely, however, that such genes could also be tested in vitro prior to their introduction into plants. We should begin immediately to consider which, if any, genes which are currently available could be used for these purposes. A significant need for these projects is a generalizable, reliable in vitro test system, preferably based on an E. coli expression system.

4. Cloning gene regulatory regions for genetic engineering of resistance genes for tissue specific expression.

During this part of the discussion, it was brought out that in addition to introduction of genes that could reduce levels of aflatoxin in plants, it is necessary to regulate the expression of these genes in the appropriate tissues at appropriate levels. Some potential regulatory regions discussed consisted of promoters that could either constitutively express genes, express genes in a tissue specific manner, or even express genes in response to wound induction. Depending on the type of gene introduced, different types of promoters may result in expression of the newly introduced gene in specific tissues at suitable levels. The consensus of the group seemed to be that more specific expression/responses to reduce levels of aflatoxin would be most desirable.

Currently, very few genes with tissue specific expression (or other functions governed by gene regulatory regions) have been cloned from the crops subject to aflatoxin contamination. It is likely, however, that heterologous regulatory DNA sequences (from other crops) would function in a regulated fashion in crops subject to aflatoxin contamination; for example, several genes with tissue and developmental specific expression have been cloned from soybean which could contain valuable promoter regions for engineering resistance genes. A case in point is the cottonseed storage protein gene which was expressed in a regulated fashion in a heterologous host (tobacco). However, very few examples of regulated gene expression in transgenic heterologous hosts are available due to the relative newness of transformation/regeneration systems for crops subject to aflatoxin contamination.

Another concern is determination of where and when defensive genes should be expressed to achieve the greatest effect. At present, very little is known about the routes of "infection" or attack by A. flavus into living (metabolically active) peanut tissues. Basic studies are badly needed to elucidate these routes of entry to allow rational design of regulated expression cassettes for defensive genes.

Identification of Resistance to Pre-harvest Aflatoxin Contamination in Peanut. C. C. HOLBROOK[1], D. M. WILSON[2], M. E. MATHERON[3], W. F. Anderson[1] and M. E. Will[2]. [1]USDA-ARS, Tifton, GA; [2]Univ. of Georgia, Tifton, GA; [3]Univ. of Arizona, Somerton, AZ. One of the major limitations in breeding for resistance to pre-harvest aflatoxin contamination (PAC) in peanut (Arachis hypogaea L) has been the difficulty in reliably measuring resistance in the field. The objectives of this research were to develop reliable and efficient field screening techniques and to begin screening the peanut core collection for sources of resistance to PAC. Three systems were examined using Florunner plots at Yuma, Arizona in 1990 (normal planting date, late planting date, and normal planting date with shading during the stress period). Results showed that the best of these systems was a normal planting date without shading. However, the samples had a low mean contamination (228 ppb), unacceptably large error variance (C.V.=223%) and unacceptably large frequency of escapes (43%). It was proposed that the screening technique could be improved by using subsurface irrigation to maintain plant life while imposing an extended drought stress period on the pods. Using this system in 1991, the mean was increased to 1,167 ppb, the C.V. was reduced to 102% and only 4% of the samples were escapes. A two year study examining ten inoculation techniques at Tifton, GA, demonstrated that Aspergillus parasiticus should be applied at midbloom (60 DAP) using corn as an organic carrier. Results from Tifton in 1991 indicated that portable greenhouses which can be moved using tractors, can be used to greatly expand the field space suitable for screening for resistance to PAC. One hundred and ninety accessions from the core collection were evaluated and two showed a high level of resistance to PAC.

Related Journal Publications

Holbrook, C. C., W. F. Anderson and R. N. Pittman. 1993. Selection of a Core Collection from the U. S. Germplasm Collection of Peanut (Arachis hypogaea L.). Crop Science 33:(Accepted for publication).

Will, M. E., C. C. Holbrook and D. M Wilson. 1993. Evaluation of Aspergillus parasiticus inoculum formulation and application timing in screening of peanut genotypes for resistance to pre-harvest A. flavus group infection and aflatoxin contamination. Peanut Sci. 19:(In Review).

A Greenhouse Technique for Evaluating Peanut Germplasm for Resistance to Pre-Harvest Aflatoxin Contamination W.F. Anderson, C.C. Holbrook, D.M. Wilson. USDA-ARS and Univ. of Georgia, Tifton, GA.

An effective greenhouse screening method for Aspergillus flavus invasion and aflatoxin accumulation is necessary for screening individual peanut plant from segregating material or progeny from genetically transformed peanut. Two experiments were conducted to assess a modified greenhouse screening technique for pre-harvest aflatoxin resistance, to assess aflatoxin accumulation for peanut genotypes reported to have some aflatoxin resistance, and to investigate any variability of segregating progeny from crosses of reported resistance genotypes with an adapted cultivar (NC-V11). A greenhouse method was devised that allows plants to grow normally while pods are formed in an extremely dry environment to enhance Aspergillus sp. invasion and aflatoxin accumulation. Invasion of hulls ranged from 40% to 95% among genotypes over the two experiments while seed invasion averaged over 90% for all genotypes indicating the success in inoculation techniques. Means for aflatoxin accumulation were 23,000 ppb and 47,000 ppb for the two experiments, respectively. Significant differences ($p=.05$) were found between genotypes in both experiments, though great variability remains within genotype. Tifton-8 and the cross of Tifton-8 and NC-V11 had the highest levels of aflatoxin accumulation. AR-4 (log10 toxin = 1.77) and the AR-4 x NC-V11 cross (log10 toxin = 2.16) had the lowest mean aflatoxin levels for experiment 1 and experiment 2, respectively. Correlation coefficients of seed and hull invasion with aflatoxin accumulation ranged from 0.18 to 0.34. Results indicated that this technique is useful in identifying resistance to aflatoxin accumulation. Currently known resistant genotypes are not adequate to eliminate or dramatically reduce aflatoxin in peanuts. Research is continuing to identify genotypes with high levels of resistance.

# Resistant Varieties

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The most practical preharvest management strategy would be the development of resistant peanut varieties. Although peanuts are highly resistant to aflatoxin contamination under growing conditions with adequate moisture, this resistance breaks down under severe and prolonged drought stress. Recent studies provide strong evidence that stilbene phytoalexins in peanuts are an important natural resistance factor in preharvest aflatoxin contamination of peanuts. This evidence included the facts that (1) stilbenes are produced naturally in field-damaged peanuts; (2) stilbenes possess biological activity against Aspergillus flavus and A. parasiticus; and (3) although invasion of peanuts by these fungi can occur under any condition, aflatoxin contamination does not occur until peanuts lose the capacity for phytoalexin production as a result of drought-induced kernel dehydration. Effective resistance to preharvest contamination could be achieved if phytoalexin production continued at lower water activities. Also, more effective resistance may be achieved if varieties are selected that produce those specific phytoalexins with the highest biological activity against the toxin-producing fungi.

Studies are currently under way to study these strategies and other factors related to the feasibility of enhancing the natural resistance of peanuts to preharvest aflatoxin contamination.

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Establishment of a Phytoalexin-Producing Peanut Seed Culture System

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An *In vitro* seed culture system was established to grow peanut seed of different maturities viz., white, yellow, orange, brown, black, using a modified Murashige and Skoog medium. Under this system peanut seed of yellow orange, brown and black maturity categories grew to maturity as measured by increase in their size and germinability. *In vitro* cultured seeds produced significant amount of phytoalexins following their inoculation with *Aspergillus* spp. while the non-inoculated sterile controls failed to produce any phytoalexins. Exposure of seed cultures to water stress using various concentrations of mannitol ( 0 to 2 M ) and polyethyleneglycol 8000 (0 to 40%) caused a significant decrease in their phytoalexin producing ability and more fungal growth compared to the non-stressed controls. This would indicate that *in vitro* grown seeds responded to water stress similar to the field grown peanuts by loosing their ability to produce phytoalexins and increased susceptibility to fungal development.

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Aflatoxin Elimination Workshop
Fresno, California, November 1-3, 1992

# EVALUATION OF COMMERCIAL MAIZE GENOTYPES FOR RESISTANCE TO AFLATOXIN CONTAMINATION OF THE WHOLE GRAIN.

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The initial objective of this cooperative research is to evaluate the effectiveness of a new screening approach for identifying sources of genetic resistance among maize inbreds and hybrids. A reliable screening method is critical to the success of any conventional maize breeding program. Thirty-four (34) commercial maize hybrids and twenty-five (25) inbreds, including 12 identifiable foundation lines, were evaluated for their resistance/susceptibility to aflatoxin contamination of the grain by Aspergillus flavus at Bloomington, IL (1990-1991) and Union City, TN (1990). Maize ears in the late-milk to early-dough stage of kernel maturity are wound-inoculated at three equidistant points along the ear with a mixed conidial suspension (1 x 10 6 conidia/ml) consisting of three aflatoxin-producing strains of A. flavus NRRL 6412, NRRL 6444 and NRRL 3557. Inoculation is accomplished by inserting a pipe cleaner contaminated with A. flavus spores into each of three holes (3-4 mm diam.) punched through the husk with a drill bit that also damages the underlying kernel(s). At harvest we inspect each wound site for A. flavus sporulation and then remove the pipe cleaner and any kernels damaged by the drill bit. Approximately twenty (20) non-damaged whole kernels surrounding each of the three wound-inoculation sites are removed from the ear and pooled with similar sets of kernels from ears within a given planted row. Each of these pooled row samples are then examined for numbers of BGYF kernels and aflatoxin (ppb). Elimination of the individual wound-inoculated kernels from our sample removes a source of highly aflatoxin-contaminated grain that has little relevance to the resistance of maturing grain to A. flavus infection and subsequent aflatoxin contamination. In 1990 and 1991, hybrids planted in Bloomington, IL showed average aflatoxin values (4 replicate rows) ranging from 26 ppb to 590 ppb and 56 ppb to 642 ppb respectively, while the same hybrids planted in Union City, TN (1990) averaged 130 ppb to 1870 ppb. Two hybrids with the best overall aflatoxin ranking (years & locations) shared a common inbred that was not a parent of any other hybrid we tested. The hybrid B73 x MO17 ranked among the most aflatoxin susceptible hybrids tested while the inbred B73 ranked among the most aflatoxin susceptible inbreds. Our second objective was to learn if the rank assigned for resistance to A. flavus/aflatoxin might be linked to ear-rot resistance rankings for other fungal pathogens (e.g. Diplodia maydis, Fusarium moniliforme and Gibberella zeae). No consistent patterns of association have emerged from the data. The results from 1992 field trials in Illinois and Tennessee will be evaluated with these earlier results as we conclude this initial phase of of our Cooperative Research and Development Agreement No. 58-3K95-M-67.

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# Identification of Molecular Markers Associated With Genes for Preharvest Resistance in Corn to *Aspergillus flavus* and Aflatoxin Production

**Progress Report**

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University of Illinois has an extensive collection of inbreds that are being screened for resistance to *Aspergillus flavus*. Inbreds are being screened as F1 crosses to B73 and/or Mo17. By screening for resistance in F1 crosses it is hoped that resistance which is dominant can be identified. In 1990-92 we developed an inoculation technique. The inoculator is a pinboard with seven rows of 25 pins with 0.7 cm of the point end exposed. Pins are spaced .4 cm apart and in the center of the pins is a large needle through which 5 ml of a spore suspension ($1 \times 10^6$ conidia per ml in 1991 and $1 \times 10^8$ in 1992) is injected under the husk. The inoculator is aligned with the ear axis and the pins pushed through the husk and into the kernels. In 1991 we evaluated 1,189 F1 crosses with Mo17 and 978 crosses with B73 in two replicates. Twelve to 18 plants in each replicate were inoculated 20-24 days following pollination. Thirty to 40 days following inoculation ears were evaluated for the amount of rot (1-10 rating scale with 1=10%, 2=20%.., 10=100% inoculated area rotted). Seventeen F1 sources of resistance from crosses with Mo17 and 18 from crosses with B73 were selected for further study. Four of the sources of resistance provided resistance in F1 crosses with both B73 and with Mo17. The remainder of inbreds provided resistance to only B73 or Mo17, or some inbreds were not evaluated as F1 crosses with one of the two susceptible inbreds. In 1992 the resistant inbred, susceptible inbred (B73 or Mo17), F1, F2, and backcross to susceptible generations were evaluated for all 35 sources of resistance. For 10 sources of resistance we also evaluated 80 to 100 F3 families. Evaluations were completed as of 10/27/92. We have tentatively selected seven inbreds crossed with B73 and 13 inbreds crossed with Mo17 for further study. Two sources of resistance will be studied with both Mo17 and B73. At this time most of the data has been entered into the computer for analysis. The final selection of sources of resistance to be used in continued studies will depend upon analysis of 1992 results. In general, those inbreds identified to have dominant resistance that segregates in the F2 and/or F3 and backcross susceptible generations will used in future studies.

In 1990 and 91 we also evaluated 15 commercially used hybrids. A number of the hybrids have wide use in the Corn Belt and others were selected because they were high in aflatoxin with natural conditions in 1988. Inoculation of these hybrids was performed at different number of days after flowering. In general, those hybrids that were a problem in 1988 had the highest disease score following inoculation. The best rating for a commercial hybrid was five (5 = 50% inoculated area rotted). The mean of all hybrids was six. Most of our better sources of resistance in the F1 with Mo17 or B73 are rated 2-4 (2=20%; 4=40% inoculated area rotted). It is not known at this time if this is enough resistance to produce acceptable control *A. flavus* in corn.

Inbred sources of resistance have been tested for RFLP polymorphism with Mo17 and B73. Probes polymorphic between resistant and susceptible inbreds have been identified. Polymorphic probes are necessary for RFLP mapping analyses of inoculated F3 families.

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HEAT STRESS AND AFLATOXIN SUSCEPTIBILITY IN CORN

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Major outbreaks of aflatoxin in the Midwestern United States are associated with exposure of the corn crop to extreme heat stress. We have hypothesized that the heat stress increases the susceptibility of corn to Aspergillus flavus infection resulting in aflatoxin contamination of the corn grain. Based on this hypothesis, we have taken three approaches to the improvement of aflatoxin resistance in corn. These are: 1) determine the physiological response of developing corn kernels to heat stress so that the mechanisms of increased susceptibility or resistance during heat stress might be determined and used in a marker assisted breeding program, 2) test directly in the field the hypothesis that heat stress increases aflatoxin susceptibility in corn, and 3) breed for increased stress resistance in corn and test if increased stress resistance results in increased aflatoxin resistance.

To determine the physiological response of developing corn kernels to heat stress, we have grown hybrid corn kernels known to be aflatoxin susceptible (B73xMo17) in culture, isolated from the ear, at controlled temperatures. We have found that optimal growth of this hybrid occurs at 25$^{o}$ C. At higher temperatures, we have found that the activity of a key enzyme controlling starch synthesis, ADP-glucose pyrophosphorylase, is dramatically reduced, as are other enzymes involved in grain filling, such as aldolase, asparate aminotransferase and invertase. The decreased rates of grain fill can be attributed to the decreased activity of these enzymes. But more importantly to fungal resistance, we have found that activities of acid phosphatase and other acid hydrolases associated with disease resistance, are reduced in heat stressed kernels. We suspect that these and other "pathogen response proteins" are key to aflatoxin resistance.

To test the relationship between heat stress and aflatoxin susceptibility, we have grown the same aflatoxin susceptible corn hybrid and suspected heat resistant Arizona Arid Environment corn lines in the consistently stressful environment of Weslaco, Texas, as well as in less stressful environments of Union City, Tennessee, and Bloomington, Illinois, inoculating ears with A. flavus. As of yet we have only analyzed Texas samples for aflatoxin. Whereas aflatoxin was not significantly different in any genotype in kernels adjacent to the wound site, Arizona lines all had significantly lower aflatoxin in kernels remote from the wound site. Although this does not represent the type of aflatoxin resistance we need in commercial corn, the Arizona lines appear to have some improved aflatoxin resistance over the susceptible control.

Crosses between corn belt lines and the previously described Arizona lines have already been made and were evaluated last year for heat stress resistance in a greenhouse experiment. Those lines that were most resistant to aflatoxin in Texas were also the most heat stress resistant in the greenhouse experiments. Some crosses derived from the heat resistant Arizona lines also appear to be more stress resistant.

To conclude, we believe that heat stress affects many aspects of corn physiology and that the effects decreasing yields may be quite different from the effects that cause increased disease susceptibility. Although our results on the effects of heat stress on aflatoxin susceptibility are not yet as conclusive as we would like them to be, there are some clear cut trends suggesting that the identification of heat stress resistance in corn germplasm may also lead to aflatoxin resistance in corn.

# Breeding for Resistance - Where We Are?

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The procedures that have been used to identify resistance follow known productive approaches. That is, develop inoculation techniques to get a uniform and hopefully high level of disease expression and then evaluate the genotypes by some method(s) for level of disease expression. Working with *Aspergillus flavus* provides a few unique challenges [e.g. weak pathogen, can't visually evaluate for resistance, seed being evaluated is genetically the next generation (except for the pericarp), sampling errors, etc.]. Corn genotypes resistant to kernel infection by *A. flavus* and/or aflatoxin contamination of the grain have been identified. Incidence of kernel infection in resistant genotypes typically are from 1/3 to 1/2 that found in susceptible genotypes. Generally, the difference between the two groups will be greater for aflatoxin contamination than for kernel infection. We have released two inbreds, Mp313E and Mp420, for resistance to kernel infection by *A. flavus* and Neil Widstrom in Georgia has released a population, GT-MAS:gk, for resistance to aflatoxin contamination. Thus, present procedures have been productive and should not be discarded. However, it is time to include some additional considerations in our research planning. Many of the tests that have been conducted are with hybrids and these are allowed to open pollinate in the test area. Under these conditions, the female portion of the endosperm and embryo of the seed that we evaluate is segregating as a $F_2$ population and we don't know the genotype of the male. Thus, if we have an immediate effect of the male expressed in the endosperm and/or embryo, we are measuring the effect of a given sample of pollen that falls on the silks of plants in a given plot and these samples of pollen can and will differ among plots in a given test. Testing of inbreds rather than hybrids creates some problems because of the vigor and yield of inbreds but probably should be done. We need to self our material to be evaluated or prove that the source of pollen is not important. Additionally, we need to expand our vision because there may be ways of testing for genotypes with resistance to aflatoxin contamination other than percent kernel infection or aflatoxin analyses. There are many parts of the plant including roots, leaves, silks, and kernel tissues (pericarp, endosperm, and embryo); and many evaluation procedures including aflatoxin contamination, incidence of kernel infection, colony diameter, sporulation, spore germination, and tissue consumption by the fungus. Surely, there is a combination of plant part and measurement techniques that will give us a rapid, inexpensive, and precise way to identify resistant genotypes.

# INHIBITION OF AFLATOXIN BIOSYNTHESIS BY FACTOR(S) DETECTED IN CORN KERNELS

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Kernels from two maize lines, tested for preharvest and postharvest resistance to aflatoxin contamination by *Aspergillus flavus* in a previous study, showed significant postharvest resistance to aflatoxin contamination in the present study, but showed no significant inter-line variation in this resistance. Kernels from either line also retarded the growth of *A. flavus*. To further test this resistance phenomenon, kernels from these lines were autoclaved, wounded in the endosperm or embryo, acetone-washed, or crushed prior to inoculation with *A. flavus*. All assays were incubated under conditions favorable to kernel germination. Resistance to aflatoxin contamination was lost in kernels that were autoclaved, crushed or embryo-wounded. Resistance to aflatoxin contamination persisted in kernels that were acetone-washed or endosperm-wounded, despite the fact that the exposed endosperm supported good fungal growth. Results suggest that postharvest resistance to aflatoxin contamination in these two lines is related to activities of the living corn embryo. Also, a fungal antigrowth factor, separate from the antitoxigenic factor, may be active in tested kernels.

# Investigations of Potential Defensive Chemicals in Corn Pericarp

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ABSTRACT

Esters of sterols and cinnamic acid derivatives comprise a group of compounds found in seeds of the cereal grains corn, wheat, rye, triticale and rice. Several aspects of these compounds suggested that they might function as protective compounds for pathogens, either fungi or insects, in corn. I. The principle forms of the compounds have as the sterol portion of the ester either cyclic triterpenes (rice; Evershed et al., J Chromatog 440:23/1988) or saturated forms of campesterol and sitosterol (corn, wheat, rye, triticale; Seitz, J Ag Food Chem 37:662/1989), which are forms of sterols not characteristic of membranes in any of the these plants. 2. The compounds appear to be strongly localized in the inner pericarp, including the aleurone layer (Seitz, Cer Chem 67:305/1990). 3. The cinnamic acid derivative (CAD) has been reported to be either p-coumaric or ferulic acid which can be involved in various aspects of resistance (Friend, Rec. Adv. Phytochem. 12:557/1979). 4. Preliminary work suggested that there could be as many as a dozen or more compounds of this type in corn pericarp, suggesting the possibility of natural selection for forms with targeted specificity. A set of experiments was performed to test the hypothesis that one or more of these compounds might affect the level of invasion by some corn pathogens.

Preliminary work suggested that the steryl CAD composition was more complex than previously reported and an efficient method of separating amounts of compounds sufficient for bioassay was devised. Esters were extracted with hexane and fractionated by column chromatography, Chromatotron, semi-preparative HPLC and analytical HPLC. An HPLC solvent system was devised which gives better separation of compounds than found in the literature: Acetonitrile:n-Butanol:Acetic Acid:Water (93:4:2:1 v/v/v/v). UV spectra indicate a number of uncharacterized sterol CAD compounds present a low levels in the ester fraction.

Bioassays of pure ferulate esters and the complex mixture from both corn and rice show no appreciable effect at 1000 ppm on the growth of *Aspergillus flavus* or at 100 ppm on the growth of *A. flavus* or two other common pathogens of corn ears: *Fusarium moniliforme* and *Diplodia maydis*. When the same compounds were tested against *Sclerotinia sclerotiorum*, however, appreciable inhibition of germination and germ tube growth was shown by the total steryl CAD ester fraction from corn and, to a lesser extent, rice bran oil. Two of the major esters present in the corn mixture; stigmastanyl ferulate and campestanyl ferulate, showed little activity--the former showed none and the latter only a small amount. The indication, therefore is that either there is a synergistic effect or that one or more of the minor components is quite active against *Sclerotinia*. The same set of compounds had no effect on corn earworm larvae or driedfruit beetle larvae or adults, however a nonpolar fraction from corn bran showed significant inhibition of growth and feeding in both insects when tested at 3X the level in bran and further characterization of this fraction is underway.

In sum: esters of cinnamic acid derivatives and saturated sterols or cyclic triterpenoids appear not to affect resistance of corn to growth of some common fungal and insect pathogens but they may have significant activity against other fungi.

# SOURCES OF GENETIC RESISTANCE TO AFLATOXIN CONTAMINATION IN ALMOND

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## ABSTRACT

Nearly all North American production of almonds occurs in California. Almond is an economically important crop due to the high value of the raw and processed product and because approximately 60% of the $500 million crop is exported, mainly to western Europe. Very low to zero tolerance levels in these markets have prompted the search for genetic/management strategies to eliminate contamination as even a few contaminated nuts can lead to product rejection.

Preliminary data supports the potential for three separate sites for inhibition of fungal infection in the maturing almond fruit: the seed coat, seed cotyledon, and the endocarp (shell). In-vitro colonization and sporulation of aflatoxigenic *Aspergillus flavus* Link on both intact and wounded seed was evaluated for a selection of almond (*Prunus dulcis* [ Mill.] D.A. Webb.) cultivars. Barriers to fungal development were identified in the intact seed coat as well as seed cotyledon tissue. The seed coat barrier was expressed as the absence of fungal colonization for up to 3 days following the inoculation of intact seed. Seed coat resistance was observed to be uniformly high for all cultivars tested. Cotyledon resistance, which was expressed as a lower rate of disease development, was identified only in the cultivars 'Ne Plus Ultra', 'Ruby' and 'Carion'.

Considerable variation was also observed for the integrity of shell seal for different genotypes, as well as their susceptibility to navel orangeworm [*Amyelois transitella* (Walker)]. Only completely sealed shells conferred resistance to the navel orangeworm and very small endocarp fractures may even facilitate its survival.

Gradziel, T. M. and Dechun Wang. Susceptibility of California Almond Cultivars to Aflatoxigenic *Aspergillus flavus*. Hort. Science. (In Press).

**Summary of Panel Discussion: Can We Identify and Utilize Aflatoxin Resistance in Germplasm?**

Panel Members: C. Holbrook, D. Doehlert, T. Rocheford, G. Scott, D. White and D. Whicklow

The discussion was opened with some general comments on the requirements for development of cultivars with resistance to aflatoxin contamination. One requirement is genetic variance (genes) for resistance. Another requirement is reliable and efficient screening techniques which can be used to identify plants which contain these resistant genes. Since plant breeding is long-term research, the third requirement is sustained financial support.

The importance of a good screening technique was illustrated with some brief comments on progress of some aflatoxin research conducted in the late 70's and early 80's. The work involved evaluating peanut germplasm for Aspergillus colonization of rehydrated peanut seed in petri dishes. Although genotypes were identified which consistently supported less colonization under these conditions, they were not resistant to aflatoxin contamination under natural field conditions. Current screening of peanut germplasm is based on measuring aflatoxin contamination in field grown peanut.

A question was raised about the usefulness of using drought tolerance or avoidance to reduce the aflatoxin problem in peanut. The concept appears to have merit, however, it is not known if a drought tolerant plant would provide enough moisture to the pods to prevent aflatoxin contamination under drought stress conditions. Research is ongoing to answer this question.

This section was concluded with a discussion of the error variance of aflatoxin data. It was pointed out that the C.V.'s reported during the peanut presentation were much higher than would be acceptable with a trait like yield. A question was raised about whether this should be of concern. It was reported that the C.V.'s for aflatoxin contamination in corn are also higher than would be acceptable for yield. The level of error variance is always a function of the type of data and assessment method(s). C.V.'s are notoriously high for data on resistance to soil-borne pathogens. This is probably due to the nonrandom dispersal of pathogens and the complexities of the interactions between host, pathogen and environment. In spite of the high C.V's, breeding progress has been made with many soil-borne pathogens. Given adequate genetic variance, the high C.V.'s associated with aflatoxin contamination will not preclude breeding progress in developing resistance to aflatoxin contamination.

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